HAEMOPOIESIS
Cell Production and its Regulation

CIBA FOUNDATION SYMPOSIUM

ON

HAEMOPOIESIS

Cell Production and its Regulation

Editors for the Ciba Foundation

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and

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PREFACE

This volume contains papers and discussions of a small international conference held at the suggestion of Professor J. M. Yoffey, who thought that quantitative aspects of blood cell production could profitably be discussed by experts in haemopoiesis, however many other haematological conferences might have been held in recent years. The Director of the Ciba Foundation readily concurred in this view, but on condition that Professor Yoffey would act as Chairman. Other members, the Foundation, and it is hoped the readers of this volume will be grateful to Professor Yoffey for his initiative and for his modest direction of the proceedings.

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List of those participating in or attending the Symposium on "Haemopoiesis: Cell Production and its Regulation" 2nd–4th February, 1960

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THE LYMPHOMYELOID COMPLEX

J. M. Yoffey

Department of Anatomy, The University, Bristol

The evolution of the lymphomyeloid tissues

The lymphomyeloid complex is the total mass of tissue concerned with the formation of the blood cells, and also to a large extent with antibody formation. It is present throughout the vertebrate series, but shows a sequence of evolutionary changes of whose precise significance we are not as yet aware. In the cyclostomes it is difficult to identify separate lymphoid tissue, and the lymphomyeloid tissue is found in two situations. The first is under the mucous membrane of the intestine, where it forms the spiral valve, and the second is among the tubules of the mesonephros. Neither lymph glands nor spleen are present, while even the existence of rudimentary thymic tissue has been disputed. Lymphoid and myeloid elements are closely intermingled in the cyclostomes, and here therefore one may speak of true lymphomyeloid tissue.

In the fishes, thymic tissue begins to be more clearly developed in relation to the branchial epithelium, and may indeed project onto the surface of the body (Deanesly, 1927; Hafter, 1952). The spleen also now clearly develops as a separate organ, and probably is the result of the dorsal migration of the tissue of the spiral valve (Jordan and Speidel, 1930). However, some lymphoid tissue always seems to remain closely associated with the intestinal mucous membrane, and the persistence of this relationship may possess a significance which we do not as yet understand. The chemical links which exist between the intestinal epithelium—possibly also the kidneys—and the formation of

blood cells may date back to their early juxtaposition in vertebrate evolution.

The next big step in the evolution of the lymphomyeloid complex occurs in amphibia, when the bone marrow first appears as a clear-cut entity. It is however only in birds and mammals that one finds what we have come to regard as the most typical lymphoid tissues, in the form either of lymph glands, as in mammals, or of more diffuse lymphoid deposits, as in birds (Kondo, 1937; Biggs, 1957). We do not know why there should have been this relatively late formation of lymphoid tissue in the two main groups of warm-blooded vertebrates. Possibly it may have been due to the greater liability to bacterial infection and the need for increased antibody formation.

The dissociation of the lymphomyeloid tissues

Whatever the reason for all these evolutionary changes, a broad trend seems to emerge Vertebrates start out with a single lymphomyeloid tissue, and this gradually separates into distinctive lymphoid and myeloid components. In mammals the dissociation appears to be complete, and this, if true, would be a most intriguing phenomenon. But further examination suggests that the separation of lymphoid from myeloid tissues is not as thorough as would appear at first sight.

The lymphoid tissues continue to show a variable tendency to develop myeloid elements This is very striking in the myeloid metaplasia which is occasionally noted in the lymph nodes. Though it is by no means a frequent occurrence, one need only see a single really good specimen of a germ centre whose cells are becoming granulocytes in order to refrain ever after from feeling too sure about the complete divorce of lymphoid from myeloid tissues. Myeloid metaplasia in adult life may be a pathological phenomenon, but it is seen regularly in foetal lymph nodes and thymus, while in the smaller laboratory animals the spleen is regularly the site of erythropoiesis and granulopoiesis.

Lymphoid tissues then are not as exclusively lymphoid as they might seem, since to a variable extent they may contain myeloid elements. How about the myeloid tissues? Here too, bone marrow does not contain obvious lymphoid tissue, but nevertheless has, dispersed among its myeloid elements, quite appreciable numbers of lymphocytes.

The apparent evolutionary divorce of the lymphoid from the myeloid tissues raises therefore a number of problems. Is the separation really complete? Is the lymphoid complex merely the original lymphoid tissue which has, for some reason or other, separated from the myeloid, or is it in fact a completely new development? If the former is the case, then what purpose has been served by the lymphoid tissues splitting off from the myeloid? And if they really have split off, to form a distinct structural entity, is there any functional connexion between them?

The quantitative study of lymphocyte production

The problems thus raised have proved to be among the most fascinating—and also among the most intractable—in the field of experimental biology. The present author can well recall his own feelings of bewilderment when, after a study of the spleen in fishes (Yoffey, 1929), he felt he would like to know more about lymphoid tissue in higher vertebrates. After a good deal of deliberation he finally decided to embark upon the quantitative study of lymphocyte production At that time it all seemed fairly simple. Ranvier (1875) had noted the lymphocyte content of thoracic duct lymph, an observation subsequently confirmed by many (for literature see Yoffey and Courtice, 1956). The thoracic duct seemed to be a sort of heaven-sent natural bottleneck through which most of the lymph of the body returned to the blood, and it also appeared to be the channel through which newly formed lymphocytes entered the blood. Counting the lymphocytes in thoracic duct lymph seemed therefore to be a

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simple way of estimating lymphocyte production (Yoffey, 1932-1933, 1936). The ratio between thoracic duct lymphocytes and those in the circulation was described as the Daily Replacement Factor (D.R.F.) on the assumption that the thoracic duct lymphocytes were newly formed cells. In the dog the D.R.F. was found to be about 2·5, which meant that the lymphocytes remained in the blood for about 10 hours. Data which

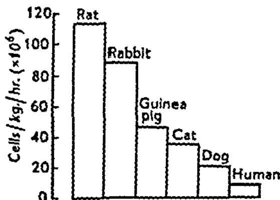


FIG 1 Thoracic duct lymphocyte output in different animals (From Yoffey and Courtice (1956, fig. 83, p. 343) The original figure was kindly prepared by Dr W O Reinhardt)

have since accumulated (Fig. 1) from other sources suggest that the lymphocyte content of thoracic duct lymph tends to vary inversely with the size of the animal The rat, for example, seems to have a much higher lymphocyte content than man. There may be other important species differences, so that generalizations based on a single species may not be universally applicable.

Difficulty of estimating lymphocyte production

The thoracic duct is not the only channel through which lymphocytes can enter the blood. They can do so through other

lymph channels, such as the right lymph duct, and irregular lymphaticovenous communications in the thorax and abdomen —these are the indirect entry lymphocytes—or else they may pass into the blood stream directly through the walls of blood capillaries in lymphoid tissue (Schulze, 1925; Ehrich, 1929). These direct entry lymphocytes we are not able to measure. Estimates of lymphocyte production based on the thoracic duct alone are therefore minimal.

We do not know why some lymphocytes should enter the blood directly, while others pass into the lymph channels and so enter the blood indirectly. It may be that the direct entry lymphocytes are more actively motile than the other groups. In that case it could well be that their greater motility would result in their leaving the blood stream more quickly. This, if true, could readily account for the two different lymphocyte populations in the blood which some workers have postulated, one remaining in the blood a good deal longer than the other (Ottesen, 1954). Whether this be so or not, it would appear rash to base generalizations about the life cycle of the lymphocytes solely on the thoracic duct lymphocytes, which may represent a special group of cells.

The obvious way of measuring total lymphocyte production is to study it at the source, in the lymphoid tissues themselves; in this way one could expect to ascertain both the direct entry and the indirect entry lymphocytes The pioneer studies in this field we owe to Kindred (1938, 1940, 1942), who estimated lymphocyte production by making mitotic counts in lymphoid tissue. Andreasen and Christensen (1949) later investigated mitotic activity in isolated nuclei of lymphoid organs. An obvious extension of this method is the use of radioactive isotopes which become specifically incorporated into newly formed deoxyribonucleic acid (DNA), which can then be measured either in radioautographs or following chemical extraction (Andreasen and Ottesen, 1944, 1945; Fichtelius, 1953, Gyllensten, Ringertz and Ringertz,

1956; Hamilton, 1957; Walker and Leblond, 1958; Yoffey, Hanks and Kelly, 1958; Perry *et al.*, 1959). Tritiated thymidine is an exciting recent addition to the labelled compounds at our disposal (Bond *et al.*, 1958; Firket, 1958; Yoffey, Everett and Reinhardt, 1958; Cronkite *et al.*, 1959; Schooley, Bryant and Kelly, 1959; Everett, Reinhardt and Yoffey, 1960; Everett *et al.*, 1960).

Kindred (1940), on the basis of mitotic counts in 15- and 20-day-old albino rats, concluded that the thymus was the most active producer of lymphocytes in the body; Andreasen and Ottesen (1944) and Andreasen and Christensen (1949) were in broad agreement with this view. Following the observations of Boivin, Vendrely and Vendrely (1948) on the relative constancy of the DNA content of mammalian cells, Yoffey, Hanks and Kelly (1958) used the DNA turnover as a measure of cell production in the guinea pig. Table I gives the results obtained.

Table I

LYMPHOCYTE OUTPUT IN GUINEA PIGS (APPROXIMATELY 400 G WT) ESTIMATED FROM DNA TURNOVER
(From Yoffey, Hanks and Kelly, 1958, and Kelly, 1958)

Tissue	*Cells per hour* × 10⁶
Mesenteric nodes	25·0
Cervical nodes	11·0
Thymus	19·6
Spleen	14 0

Note: The thymus data were obtained in seven animals whose mean weight was 370 g and the remaining data on sixteen guinea pigs of mean weight 412 g

[illegible] based on DNA turnover or mitotic counts do not [illegible]

[illegible] and Trowell [illegible]. However Kindred (1942, [illegible]

The problem of lymphocyte circulation

In 1936 Sjövall put forward the view that the concept of massive lymphocytopoiesis was mistaken, and suggested that what was really happening was a continuous recirculation of lymphocytes between blood and lymph. His views seem to have been developed largely in relation to lymph nodes, as indeed have many other theories about the function of lymphoid tissue. Lymph nodes are comparative late-comers in evolution, and it cannot be too strongly emphasized that lymphoid tissue functioned long before lymph nodes appeared on the vertebrate scene. Sjövall compared the lymph node to a particle-containing lake, and he argued that if at any time there were more particles leaving the lake than were entering it, this did not mean that the particles were necessarily formed in the lake, but merely that they were present in it when the water passed through. It is evident, however, that if there are always more particles leaving the lake than are entering it, then the lake would rapidly empty.

The experiments of Ehrich and Harris (1942) showed clearly that, working with a single node (the popliteal lymph node of the rabbit), the afferent lymph contained about 2,200 cells per mm.^{3}, the efferent 17,000. Three to nine days after injecting typhoid vaccine into the pad of the foot, the cells in the efferent lymph—virtually all lymphocytes—averaged 67,000 per mm.^{3} (range 41,600–144,000). At the same time the node underwent marked hyperplasia This type of finding, taken in conjunction with the histological evidence of cell proliferation, seems to fit in best with the view that there is active new cell formation in the node

However, some recirculation undoubtedly occurs, for peripheral lymph always contains some lymphocytes. But these are relatively few in number, and from whatever source the peripheral lymph is obtained, whether intestine (Goodall and Paton, 1905–06; Florey, 1927; Baker, 1932–33) or limbs (Yoffey and Drinker, 1939), there are always far fewer cells in peripheral than in

central lymph. Yoffey and Drinker (1939) calculated that, in the dog, of every 30 cells entering the blood via the thoracic duct one had recirculated via the peripheral lymph. There is apparently a steady drift of small numbers of lymphocytes out of the blood capillaries, through the connective tissues of the body, and back to the lymph stream. But the number of such cells seems to be independent of the number of lymphocytes present in the blood stream.

Sjövall's "lake" hypothesis is very difficult to accept. The lymph node would be a cell reservoir, which should quickly disappear as more lymphocytes were leaving it than entering it. On the basis of a recirculation hypothesis, there seems to be only one way of reconciling the persistence of the lymph node with the disparity in cell content between afferent and efferent lymph. One must assume that the lymph node is a region where lymphocytes are continually passing from the blood stream into the efferent lymph [illegible] lymph node proper would remain *in situ*, receiving a steady addition of cells via the afferent lymph. It is difficult to reconcile all this with the evidence of active cell proliferation.

Gowans (1957, 1959), extending the earlier experiments of Mann and Higgins (1950), has revived the recirculation concept. When a fistula was maintained for four days, Gowans (1957) found that the thoracic duct lymphocytes markedly decreased while at the same time there was a fall in the blood lymphocytes, thus confirming the observations of Mann and Higgins (1950).

The interpretation of these experiments is not easy. It seems probable that the rat in the Bollman cage is far from normal. (*a*) The initial operative procedure, probably as the result of a "stress" reaction (Selye, 1937, Dougherty and White, 1944), upsets the lymphoid tissues, and produces a lymphopenia which *may* last for several days (Sanders, Florey and Barnes, 1940; Adams, Saunders and Lawrence, 1945; Andreasen and Gottlieb,

1947). In fact even anaesthesia without operation may have this effect (Yoffey, 1935). (*b*) From what we know of the effects of forced immobilization, restraint in the Bollman (1948) cage may cause a further stress reaction. (*c*) The animals lose weight. Gowans (1959), following Mann and Higgins (1950), considers the loss of weight not to have any bearing upon the results of his experiments, but in view of the well-known effects of inanition upon lymphoid tissue (Reinhardt, 1943) this is open to dispute. (*d*) The animals develop a marked neutrophilia (cf. Adams, Saunders and Lawrence, 1945; Glenn, Bauer and Cresson, 1949; Mann and Higgins, 1950). Gowans (1957) noted that "All the animals developed polymorpho-nuclear leucocytosis by the end of the experiment, the counts varying from 5,000 to 20,000 cells per c.mm. This remains unexplained." The association of neutrophilia and lymphopenia is well known, and in our own studies neutrophilia was accompanied by increased uptake of lymphocytes in the bone marrow (Harris, Menkin and Yoffey, 1956). (*e*) The animals received repeated doses of heparin. Even single doses of heparin in amounts not much greater than those given by Gowans (1957) can give rise to eosinophilia (Braunsteiner, Potuzhek and Thumb, 1959; Hamilton, 1957), in addition to possible changes in plasma constituents and capillary permeability (Yoffey and Courtice, 1956). The cumulative effect of all these factors may be considerable, the condition of the thoracic duct fistula animal in the Bollman cage may be far from normal, so that some caution should be exercised before drawing from these experiments conclusions of too sweeping a nature.

The stress effect of the operative procedure is of course common to all studies on thoracic duct lymphocytes. Working with the guinea pig Reinhardt and Yoffey (1957) endeavoured to cut down to the absolute minimum the operative str[illegible]

suction. This gave precisely the same results as far as lymph

collection was concerned, but began it much earlier, often within ten minutes of the animal being anaesthetized. Such a procedure may possibly minimize the stress response, but probably the only way of avoiding it completely would be to kill the animal instantaneously and then make mitotic counts in sections, as was done by Kindred (1938, 1940).

Lymphoid tissue after prolonged lymph drainage

On the basis of pure recirculation, the effect of prolonged thoracic duct drainage should be to cause progressive depletion and ultimate disappearance of the lymphoid tissues. Gowans (1959, Fig. 1) found in rats that during the first 24 hours after cannulation the output of thoracic duct lymphocytes was greater than the combined volume of the abdominal lymph nodes. These nodes should therefore have completely disappeared—unless, as previously noted, one adopts the improbable assumption that most of the lymphocytes in the nodes are stationary, while the cells in the efferent lymph are derived from the blood flowing through the nodes Unfortunately, no information is available about the state of the abdominal lymph nodes in these animals. The absence of such information adds a further serious difficulty to the interpretation of the chronic fistula experiments. The only evidence which seems to be available is that provided by Glenn, Bauer and Cresson (1949) They examined the popliteal lymph nodes in two dogs, each with a four-day fistula, removing one popliteal node at the beginning of the experiment. They found that the remaining node had lost weight slightly at the end of four days, but concluded that "It was not possible to correlate the decrease in lymphocyte output through the thoracic duct with the histologic findings in the popliteal nodes"

Variation in lymphocyte production

It has been known for some time that lymphocyte production is subject to considerable variation (Yoffey, 1936; Yoffey, Hanks

and Kelly, 1958). This can be readily understood if it is related to the cyclic changes which occur in lymphoid tissue (Maximow, 1927; Conway, 1937). If however the lymphocytes are for the most part recirculating, one must look for some other explanation of the great range of variation in the lymphocyte content of thoracic duct lymph.

Non-disappearance of blood and thoracic duct lymphocytes in chronic fistula

On the basis of the recirculation hypothesis, the effect of a chronic fistula might be expected to be a progressive diminution and ultimate disappearance of thoracic duct and blood lymphocytes. Instead, though it is true that they fall markedly after a while, they seem to attain a stable lower level and then persist unchanged.

Marked lymphocytes

A number of investigators have endeavoured to study the problem by following the fate of marked lymphocytes. Farr (1951), employing acridine dyes, observed transfused cells in lymphoid tissue and bone marrow. Fichtelius (1953), using thymocytes labelled *in vivo* with ^{32}P, found relatively high activity in spleen and bone marrow and then in descending order in thymus, small intestine, liver, lymph node, and lung. Keohane and Metcalf (1958), working mainly with thymocytes labelled with diaminoacridine, could recover in the rat only 5 per cent of the transfused cells in thoracic duct lymph. Gowans (1959), also in the rat, found that after transfusing thoracic duct lymphocytes labelled with ^{32}P "about 20% of the total radioactivity and about 40% of the radioactively labelled lipid in the transfused cells were recovered in the lymphocytes from the thoracic duct". This represents an appreciable number of recirculating cells—the recirculation occurring over several days—but still seems to leave a substantial number which are not recirculating.

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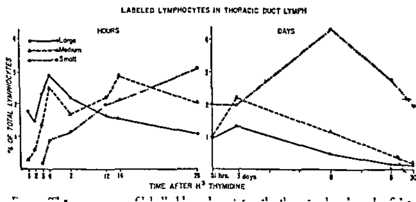
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It has been known for some time that lymphocyte production is subject to considerable variation (Yoffey, 1936; Yoffey, Hanks

change being a cytoplasmic reduction, with certain nuclear alterations in addition. The cells at the various stages of the reduction pathway constitute the Precursor Pool, and are all capable of thymidine uptake, i.e. DNA synthesis and subsequent division, except for the final small lymphocyte. Within the lymphoid tissues the great majority if not all of the small lymphocytes seem to be incapable of growth (cf. Maximow, 1909).

Following the administration of tritiated thymidine to guinea pigs, labelled lymphocytes begin to appear in thoracic duct lymph



within half an hour or even less (Fig. 2). To begin with the labelled cells were preponderantly large lymphocytes, but gradually medium lymphocytes appeared in increasing numbers, and then small lymphocytes (Everett *et al*, 1960). The Size Distribution Ratio (S.D.R.) of large, medium and small lymphocytes gradually changed. Thus during the first hour in one experiment the S.D.R. was 82·5:12·3:0, i.e. of the labelled cells 82·5 per cent were large lymphocytes, 12·3 per cent medium and 0 per cent small, at 12 hours it was 24:43:31, and at six days 8:20:69.

Working with dogs, Perry and co-workers (1959) transfused autologous lymphocytes labelled *in vitro*, but were unable to find any radioactivity in the thoracic duct lymph. Furthermore, when they gave ^{32}P to dogs, there was no sharp rise in the radioactivity of the blood lymphocytes nor was there subsequently an increase in the radioactivity of thoracic duct lymphocytes when that of the blood lymphocytes reached its peak. This they interpreted as evidence (*a*) against recirculation, (*b*) in favour of a short stay for lymphocytes in the blood. Ambrus and Ambrus (1959) found that a considerable number of transfused labelled lymphocytes could be identified in Thiry-Vella intestinal loops within a short time, as well as appearing in a number of other situations, including the thymus. As reported elsewhere in this volume, Everett and co-workers (1960) have transfused thymidine-labelled lymphocytes from the thoracic duct lymph of a donor rat and found some of them in the thoracic duct lymph of the recipient, but also in bone marrow, spleen and lymph nodes.

Experiments with tritiated thymidine

Recent studies with tritiated thymidine have thrown fresh light on certain aspects of lymphocyte production. It has long been believed that in the lymphoid tissues, beginning with a primitive stem cell—probably the reticulum cell—there occurs a series of divisions passing through lymphoblasts, large lymphocyte and medium lymphocyte stages until finally the small lymphocyte is reached. Sainte-Marie and Leblond (1958) have estimated that in the thymus something like eight mitoses occur between the stem cell and the final small thymocyte, so that each reticular cell represents potentially 128 small lymphocytes. In the rat lymph node Grundmann (1958) estimated there were only six

cent of labelled small lymphocytes, while Cronkite and co-workers (1959) in the dog did *not* find any, though they obtained 12 per cent of labelled large and medium cells. It is pertinent at this point to note that Perry and co-workers (1959) in the dog found in the thoracic duct lymph 25 to 35 per cent of large lymphocytes capable of taking up ^{32}P and presumably therefore of DNA synthesis and thymidine labelling. The relatively low percentage of labelled small lymphocytes could obviously be interpreted to mean that most of them were not newly formed, and must therefore be recirculating. Against this, however, is the fact that the labelled small lymphocytes definitely do not appear to be recirculating since they show progressive diminution of the grain count (Everett, Reinhardt and Yoffey, 1960). Thus in a number of experiments the average grain count of 25 labelled small lymphocytes fell from 42 at 13 to 14 hours, to 24 at 26 hours, 15 at 51 hours, 13 at 17 hours, 8 at 144 hours and 4·5 at 8 days. If we accept the metabolic stability of DNA, then the changing grain count must be regarded as conclusive evidence against the recirculation of the labelled cells. If the non-labelled cells are recirculating, whereas the labelled cells are not, it becomes necessary to explain an apparently radical difference in behaviour. One possible explanation would be a differential motility, as previously suggested in the case of the direct entry and the indirect entry lymphocytes. It is conceivable that most of the labelled [illegible] formed small lymphocytes are in the direct entry group, [illegible] their way into the lymph stream. [illegible] lymphocytes in the [illegible] t in with this view (Everett, [illegible]

If we endeavour to summarize the state of our knowledge so far of lymphocyte production, we would say that there is substantial new formation of lymphocytes, and also some recirculation. There are important species differences both in the total numbers of thoracic duct lymphocytes, and in the proportions of

This is what one would expect as more and more members of the precursor pool finally reached the small lymphocyte stage. However *at no time did the total number of labelled cells exceed 7 per* cent after a single injection of thymidine.

Cronkite and co-workers (1959), working with dogs, found *labelled cells in thoracic duct lymph increasing up to 12 per cent* by the fifth hour. They did not observe any labelled small lymphocytes, though otherwise the S.D.R. seemed to be tending in *the same direction as in the experiments of* Everett and co-workers (1960), namely the gradual replacement of larger by smaller cells.

The discharge of cells from the precursor pool

The use of thymidine has served to emphasize that cells are being continually discharged from lymphoid tissue at all stages of *the precursor pool. If, following the model of* Sainte-Marie and Leblond (1958), we denote the reticulum cell as R, the lymphoblast as L1, and the various other lymphocytes as L2, L3, L4, L5, *L6, L7 and L8 (L8 being the final small lymphocyte), then* R = R + L1 = 2L2 = 4L3 = 8L4 = 16L5 = 32L6 = 64L7 = 128L8.

The first question which arises is whether the subsequent history *of the cells at different stages of the reduction pathway is the same* once they pass into the lymph stream as it would have been had they remained in the lymphoid tissues. In the lymphoid tissues *an L1 cell would ultimately form 128 small lymphocytes, an L2* cell 64 small lymphocytes, and so on If the subsequent development is predetermined, then wherever these cells ultimately go after they enter the lymph and blood they would still give rise to the same number of small lymphocytes.

The second question concerns the total number of labelled lymphocytes appearing in thoracic duct lymph. The small lymphocytes should be the most numerous of all, whereas in fact in the guinea pig Everett and co-workers (1960) after a single thymidine injection could not find more than 4·3 per cent of these cells; Gowans (1959) in the rat found at the most 1·5 per

give rise to a leucocytosis of 94,000 per mm.[3] of blood. The normal neutrophil granulocytes in the blood of the standard guinea pig number 1,340 per mm.[3] and the marrow therefore possesses a reserve of mature or almost mature neutrophils which is about 70 times the number in the circulation. It is still not clear how long the neutrophils remain in the blood stream once they enter it, but estimates of their stay in the blood are tending gradually to shorten from the earlier figures of Kline and Cliffton (1952). Craddock, Perry and Lawrence (1959), for example, think that in the dog the neutrophils may remain in the blood for only six to eight hours (cf. Patt and Maloney, 1959; Mauer *et al.*, 1959). Even so the marrow still possesses a large myeloid reserve. The more immature cells in the marrow (myeloblasts, promyelocytes, myelocytes, and metamyelocytes) form about one-half the band and segmented neutrophils. If we assume that these immature cells may have an overall doubling time of 24 hours, it should apparently be possible to maintain a leucocytosis of 80,000–100,000 indefinitely.

The marrow then undoubtedly possesses a large myeloid reserve. It does not necessarily follow that this reserve can in fact be readily mobilized. Cell counts on the peripheral blood are of little value, since large numbers of neutrophils may enter the blood without any increase in the circulating cells if they are leaving the blood equally rapidly. We have therefore tried to assess the extent of the mobilization of the myeloid reserve by direct quantitative marrow studies (Yoffey, 1954, 1955; Harris, Menkin and Yoffey, 1956). The technique of leucocytapheresis* (leucopheresis, Craddock *et al*, 1955; Craddock, 1957) measures the leucocytes which can be withdrawn from the blood.

We tried the effect of Leucocytosis Promoting Factor (LPF), and found that four hours after a single intraperitoneal injection the marrow neutrophils had diminished by 1,010 × 10⁶. This

* *The ending used here and throughout the book is from the Greek aphairesis,* meaning a taking away or removal —Eds.

the different cell sizes. If the rat in the Bollman cage can be regarded as a normal animal, then the rat may have a higher proportion of recirculating lymphocytes than other animals.

The quantitative study of bone marrow

After attempting the estimation of lymphocyte production, we next turned our attention to the quantitative study of bone marrow. Following early attempts with rabbit marrow (Yoffey and Parnell, 1944) a satisfactory quantitative technique was elaborated for the guinea pig (Yoffey, 1956). Careful measurements of marrow volume by Hudson (1958, 1959) made it possible to calculate the total marrow population of the various cell groups.

The pioneer quantitative studies on the cell population of the marrow we owe to Kindred (1942); Fruhman and Gordon (1955*a*, *b*), working with rats, used a direct quantitative technique not unlike our own. Other data will be found in the papers of Osgood (1954), Suit (1957), Donohue and co-workers (1958), Patt and Maloney (1959) and Mauer and co-workers (1959).

The normal myelogram for our standard animal has a total of 1,814,000 nucleated cells per mm^3 of marrow, and of these 716,000 are granulocytes, 422,000 are erythroid, and 430,000 lymphocytes. These data have been calculated for a male guinea pig of the Dunkling–Hartley strain weighing as near 400 g. as possible. The use of this standard animal reduces to a minimum variations due to age and sex.

When det [illegible]
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circulation, and compare them with those in the marrow.

The myeloid equation

The total of segmented neutrophils in the marrow as a whole is 868 × 10^6, of band neutrophils, 1,834 × 10^6. The segmented and band neutrophils together, if discharged into the blood, would

there is not available a large reserve of red cells to meet a sudden increase in the demand. When more red cells are needed production must be increased and this (Fig. 3) means additional stem

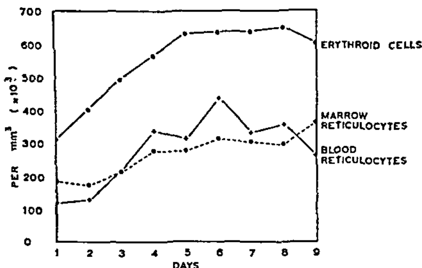


FIG. 3 Reticulocytes in bone marrow and blood, and nucleated red cells in the

cells (Yoffey, 1957); Alpen and Cranmore (1959) have recently come to the same conclusion.

The lymphocyte in bone marrow

The marrow has a high lymphocyte content. In the standard guinea pig there are 430,000 lymphocytes per mm.³ of marrow,

should have given rise to a leucocytosis of about 35,000 per mm.^{3}, whereas in fact the observed leucocytosis was almost negligible, amounting to 1,600 per mm^3. From the blood count alone it would have been impossible to know that there had been so large a discharge of neutrophils from the marrow. Band and segmented neutrophils were discharged from the marrow in equal numbers (Harris, Menkin and Yoffey, 1956).

That the marrow can rapidly discharge large numbers of granulocytes has been demonstrated by the ingenious technique of leucocytapheresis introduced by Craddock and co-workers (1955). This enables one to assess the myeloid reserve and the speed of its mobilization by measuring the number of granulocytes entering the blood. The earlier experiments, with a single acute leucocytapheresis, demonstrated that the marrow was capable of replacing the circulating blood leucocytes every two hours, and further studies by means of isotope labelling indicated (*a*) that the cells discharged were newly formed in the marrow, and (*b*) that once neutrophils had left the blood stream they did not return to it in appreciable numbers (cf. Patt and Maloney, 1959; Mauer *et al.*, 1959).

The studies of Gordon (1960), reported elsewhere in this volume, provide further evidence on leucocyte mobilization

The erythroid equation

The erythroid cells of the marrow, as had previously been noted by Kindred (1942), differ markedly from the myeloid cells. The total of nucleated red cells of the marrow as a whole is 2,616 × 10^6. If the life of the circulating red cell in the guinea pig is taken as 80 days (Everett and Yoffey, 1959), the daily requirement for new red cells would be 1,970 × 10^6, and to meet this there must be continuous new formation of red cells, since there is not a large reserve of reticulocytes in the marrow (cf. Reiff *et al.*, 1958). The erythroid cells of the marrow can just about keep pace with the need for new red cell formation. But unlike the granulocytes

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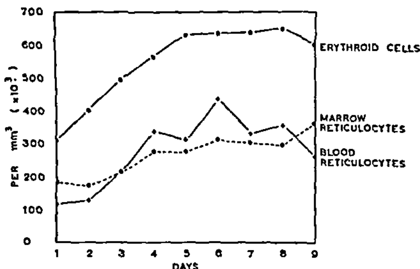


FIG 3. Reticulocytes in bone marrow and blood, and nucleated red cells in the marrow of guinea pigs at the Jungfraujoch (This graph is based on data obtained by a team consisting of Hudson, R. S Harris, P F Harris, Batten, Rogers, Reinhardt and Yoffey, 1954) The nucleated red cells of the bone marrow increase about twofold in 5 days; in exceptional instances they may increase beyond this, and reach a level of about 1,000,000 per mm³ of marrow The bone marrow of [illegible]

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regarded as haematogenous, since it was too rapid to be attributable to multiplication *in situ*.

The dual response. L : G inversion

A dual response thus occurs after the administration of LPF—or of bacterial vaccine. Granulocytes leave the marrow; lympho-

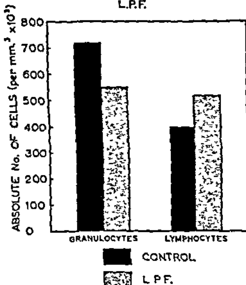


FIG 4 The dual response of the bone marrow, and the L . G (lymphocyte granulocyte) inversion Four hours after giving leucocytosis-promoting factor (LPF), marrow granulocytes have diminished while lymphocytes have increased (This figure is based on the data of Harris, Menkin and Yoffey, 1956.)

cytes are taken up by it (Fig. 4). The reverse change is frequently seen in the peripheral blood where neutrophilia is found to be associated with lymphopenia. We sometimes refer to this dual

and the marrow as a whole contains $2,660 \times 10^6$ lymphocytes, compared with 131×10^6 in the blood stream, and 373×10^6 daily entering the blood via the thoracic duct. For every lymphocyte normally present in the blood three are daily entering it via the thoracic duct and 20 are present in the bone marrow. The abundance of lymphocytes in the marrow, and the changes which they undergo, are of great interest in relation both to the lymphocytes and the bone marrow. Though it may sound somewhat of a truism, it perhaps needs to be emphasized that what happens to the marrow lymphocytes can only be determined by the study of the marrow. No amount of work directed to the lymphocyte in lymphoid tissue or lymph can tell us what is happening to lymphocytes in the marrow.

There is no evidence of lymphocyte multiplication in the marrow. The cells are virtually all small lymphocytes; medium and large lymphocytes are singularly few. In the guinea pig marrow numerous cells are seen which have been interpreted as transitional between the lymphocyte on the one hand and myeloblast and proerythroblast on the other (Yoffey, 1957). The cells which Lajtha (1959) regards as large lymphocytes are probably these transitional cells. Varying accumulations of lymphocytes are found also in the marrow sinusoids, and this has been referred to as "lymphocyte loading" (for fuller details see Yoffey, 1960).

Marrow lymphocyte reactions

It has been observed that in a number of experimental conditions the lymphocyte population of the marrow may undergo either rapid increase or decrease. Rapid increase was first noted in association with the discharge of granulocytes from the marrow, after bacterial vaccines (Yoffey, 1955) or Leucocytosis Promoting Factor (Harris, Menkin and Yoffey, 1956). Mention has already been made of the discharge of granulocytes from the marrow after giving Leucocytosis Promoting Factor. At the same time there was an increase in the marrow lymphocytes which was

erythropoietic in rats. In the guinea pig it was found to stimulate granulopoiesis. As the myeloid cells of the marrow increased (Fig. 5), the lymphocytes diminished (Osmond *et al.*, 1960).

Increased lymphocytes in marrow during hypoxia

Another lymphocyte response was observed during hypoxia. During the first few days of adaptation to hypoxia, there is an increase in the marrow lymphocytes at the same time as the nucleated red cell population is increasing (Yoffey, 1957).

The effect of sublethal irradiation

Harris (1956) followed the changes in the marrow of guinea pigs subjected to sublethal whole-body irradiation (150 r.). To begin with, both lymphocytes and erythroid cells fall to a very low level. From the second day onwards the lymphocytes steadily rise, and by the 16th day have reached a level of about 900,000 per $mm.^3$. The erythroid cells increase slowly from 6 to 14 days, and then multiply more rapidly to 50 per cent above their normal level by the 20th day. The granulocytes, after an abortive peak at 6 to 10 days, rapidly increase, and are well above their normal level by the 22nd day. From the 14th to the 20th day both erythropoiesis and granulopoiesis proceed with great intensity; during this period the marrow lymphocytes fall abruptly from 900,000 to 200,000 per $mm.^3$. This has been interpreted as indicating their transformation into stem cells of the erythrocytic and granulocytic series. There is no evidence of a massive discharge of lymphocytes from bone marrow into the blood stream, since throughout this period the blood lymphocytes remain below their normal level.

The problem of the stem cell and varying stem cell requirements

The trend of all our marrow studies has been more and more to implicate the small lymphocyte as a multipotential stem cell, a

response as the L:G (lymphocyte: granulocyte) inversion. In the present example, as the granulocytes fell the lymphocytes rose, but the opposite change may also be seen. An unexpected

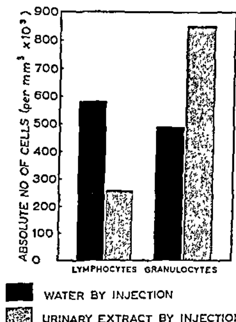


FIG. 5 Another example of an inverse relationship between marrow granulocytes and lymphocytes The granulocytes are increased and the lymphocytes diminished following the administration of a urinary extract which was erythropoietic in rats but proved to be granulopoietic in the guinea pig (This figure is based on the data of Osmond and colleagues, 1960.)

instance was encountered in some recent experiments, when guinea pigs were given daily injections for seven days of a substance, extracted from human urine, which had been found to be

gives rise to four mature erythrocytes. If we assume that in man, with a five-day maturation period, there are three mitoses instead of two, then one stem cell would give rise to eight mature erythrocytes. These estimates are not absolutely precise, and may require modification, but general considerations of this nature suggest that the marrow of smaller animals would need considerably more stem cells than that of man. Can one in fact find evidence of any significant difference in the numbers of stem cells?

When one compares human and animal marrow from this point of view, the one outstanding difference is the greater number of lymphocytes in the marrow of the smaller animals. If the lymphocyte is a stem cell, then the greater number of lymphocytes and their more obvious rôle in the marrow of small animals becomes easy to understand. If however the lymphocyte is not a stem cell, two questions require an answer. The first is:—why are more lymphocytes present in the marrow of smaller animals? The second question is: what other stem cell is there which is significantly more abundant in the marrow of smaller than of larger animals? The answer to the second question is "None". There is no clear answer to the first of these two questions.

In adult human marrow, there are usually 7 to 10 per cent of lymphocytes, and most of these seem to be inactive. During the growth period, however, the marrow has to meet not only normal replacement needs, but also to provide for the steady increase in the total number of red cells, as well as for the growth of the marrow itself. Under these circumstances, when more stem cells are needed, the only obvious change is the increase in the marrow lymphocytes, whose level is appreciably higher in infancy and childhood.

The inter-relationship between different parts of the lymphomyeloid complex

In endeavouring to study the component elements of the lymphomyeloid complex, we have been coming more and more

concept of course which is far from new. Our own work has served merely to add quantitative data which had previously been lacking.

According to *this concept the lymphocyte* is a *multipotential stem cell*, and the small lymphocyte is a *comparatively inactive* form of this cell, dormant *for long periods*, but sooner or later capable of reactivation to a new cycle of growth and development. We do not know precisely what the reactivating stimuli are, or how long the small lymphocyte may remain in its resting phase. It is possible that after a prolonged period of rest the cells may die. Further, since lymphocyte movement (McCutcheon, [illegible] to be completely random, many cells [illegible] ...sible.

[illegible] of the many puzzling p[illegible] ...ive [illegible] ...hocyte content between the [illegible] ...oratory animals. The quantitative data now [illegible] ...row light on the problem. In the smaller laboratory animals *the life of the circulating red cell is appreciably shorter than it is in man.* As against 120 days *in man* (Whitby and Britton, 1957), *the life of the red cell in the guinea pig is in the neighbourhood of* 80 (Everett and Yoffey, 1959), while in the rat and *rabbit it is 60 to 70 days or even less* (Neuberger and Niven, 1951; Berlin and Lotz, 1951; Burwell, Brickley and Finch, 1953).

Other things being equal, this difference in the life of the circulating red cells would in itself be sufficient to increase the need of additional stem cells by up to 100 per cent. But other things are not equal, for in addition to the difference in the life of the circulating red cell there are also differences in the maturation time in the marrow In the rat the total maturation time is around 48 hours (Belcher, Gilbert and Lamerton, 1954), whereas in man it is about five days (Badenoch and Callender, 1954; cf. Lajtha and Suit, 1955) If we assume that in the rat two mitoses occur between the stem cell and the mature red cell, then one stem cell

and Vos, 1957), whereas heterologous marrow merely gave rise to temporary repopulation.

Can lymphocytes confer protection?

If the lymphocyte really functions as a stem cell, then suspensions of lymphocytes should confer protection. Now while it is true that Congdon, Uphoff and Lorenz (1952) observed that thymocytes might occasionally protect irradiated mice, and while more recently Salvidio, Oliva and Pierotti (1958) have reported a protective action of heterologous lymph node cells in mice, these findings are exceptional. Campbell and Ross (1952) were unable to demonstrate any protective action of thoracic duct lymphocytes, and the consensus of opinion seems to be in general agreement with this, namely that neither lymphocytes nor thymocytes can confer protection (Cole, 1957).

Marrow transfusion stimulates regeneration of lymphoid tissue

Transfused marrow cells can seed out into the lymph nodes, liver, thymus and spleen (Ford *et al*, 1956; Mitchison, 1956; Nowell *et al.*, 1956; Porter, 1957). This could be regarded as a vascular accident. What is surprising however is that the transfused marrow cells can not only settle in lymph nodes and thymus, but also stimulate their regeneration (Congdon and Lorenz, 1954; Fishler *et al.*, 1954; Jacobson, Marks and Gaston, 1954; Porter, 1957; Urso and Congdon, 1957). One can interpret this in several ways One might assume that a common stem cell exists which gives rise to lymphocytes in lymphoid tissues, or to granulocytes and erythrocytes in bone marrow. Alternatively, one may speculate that lymphocytopoietic stem cells are normally present in the marrow, and continue to function in the same way in lymphoid tissue.

If marrow cells can stimulate the regeneration of lymphoid tissue, then marrow transfusion, if the lymphocyte can function

to the view that the different parts of the complex are in close functional relationship, and that through the blood stream there is in all probability a constant interchange of cells between them. In recent years new light has been thrown on this problem by transfusion and shielding experiments. Some of the classical work in this field has been done by two of the participants in this symposium (see Jacobson *et al.*, 1951; Jacobson, Marks and Gaston, 1955; Ford *et al.*, 1956).

Transfusion and shielding experiments

In the main, the experiments have centred round the conferring of protection against lethal doses of irradiation. Jacobson and co-workers (1951) showed that marrow cells must be provided in relatively large numbers, while Jacobson, Marks and Gaston (1955) found that marrow cells protected in proportion to the numbers given, with a maximum upper limit. They noted that haemopoietic cells from adult marrow, spleen, or embryonic liver could confer protection, and they also made the further significant observation that young donors were better than old. The experiments of Ford and co-workers (1956), using donor cells with an atypical chromosome, made it possible to identify with certainty the presence of donor cells not only in the recipient marrow, but also in spleen, thymus and lymph nodes

Homologous and heterologous transfusions

Much of the experimental work on cell transfusions has been carried out with homologous or heterologous material. Though these studies have stimulated a number of ingenious experiments it is difficult to assess their significance in terms of normal blood formation. Congdon, Uphoff and Lorenz (1952) noted that isologous transfusions were the most effective. Hirsch and co-workers (1956) went into this point carefully and concluded that only isologous marrow could promote "lasting re-generation of the host's radiation-damaged hematopoietic tissues" (cf. Bekkum

as a stem cell, would increase *the production potential for new* stem cells to a far greater extent than marrow colonization alone. This possibility is one which we are forced to consider when looking at the quantitative aspect of transfusion experiments.

The identity of the protecting cells

Alpen and Baum (1958), in transfusion experiments on irradiated dogs, give a detailed analysis of the transfused marrow cells, and point out "It is interesting to note the large proportion of the injected cells which are presumably post-mitotic and incapable of acting as regeneration potential". When one peruses their list of cells, it would seem that only myeloblasts, lymphocytes, pro-erythroblasts, and unclassified cells call for serious consideration as stem cells. The density of marrow recolonization after Alpen and Baum's marrow transfusions has been calculated elsewhere (Yoffey, 1960). Assuming that all the transfused cells settled in the marrow, there would be 85 to 140 myeloblasts per $mm.^3$ of marrow, and 600 to 1,000 lymphocytes, in either case about 1/70th of the normal marrow population. But of the two cell groups the lymphocytes seem the more numerous, and by stimulating lymphoid tissue regeneration they could rapidly increase their numbers. Myeloblasts on the other hand would have to increase in the marrow very quickly indeed to be of any use. In normal marrow mitoses of myeloblasts are infrequent, but we have no information about the myeloblasts in post-transfusion marrow.

Marrow shielding

Marrow transfusions—as also lymph node, spleen or thymus—almost certainly introduce into the blood stream many cells which do not normally obtain access to it. The most physiological type of experiment is that in which the animal is protected by shielding part of its marrow. This avoids all immunological complications, while at the same time cells which escape from the intact shielded marrow can be presumed to be such as may do so

in the normal animal. In the subsequent discussion it is assumed that the protection is due to cells, not to a humoral agent. If this assumption should prove to be unwarranted, then the argument is invalid.

From the experiments of Kaplan and Brown (1952), Nagareda and Kaplan (1955) and Weymouth and co-workers (1955) it is clear that merely shielding the marrow stimulates the regeneration of thymus and lymphoid tissue. The studies of Weymouth and co-workers (1955), comparing the thymic regeneration in animals with and without thigh shielding, suggest that there may either be a migration of small lymphocytes from marrow to thymus, or the passage of some factor which stimulates the formation of small lymphocytes. "The thymocytes which first reappear during the phase of regeneration resemble large rather than small lymphocytes, with a correspondingly greater diameter and distinctly more cytoplasm. In the shielded animals, this cell type is gradually replaced over a period of several days by the typical thymocyte or small lymphocyte and concurrently the mass of the gland increases to normal levels. In the unshielded irradiated mice, however, the large thymocyte tends to remain the dominant cell-type for an indefinite interval" (cf. Gregoire and Duchateau, 1956).

Lymphocyte loading of marrow sinusoids

The view that it is small lymphocytes which thus migrate from marrow to thymus—and lymph nodes—is supported by the distribution of lymphocytes in bone marrow They may be found either scattered throughout the marrow parenchyma (Fig. 6) or in the marrow sinusoids. The latter may possibly be in stagnant sinusoids (cf. Brånemark, 1959) forming a reserve which can rapidly pass into the circulation when needed. It should be noted that from the point of view of passage of cells through their walls, the venules and large draining veins of the marrow seem to function exactly as the sinusoids, having walls of equal thinness.

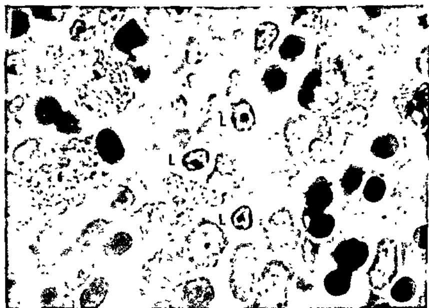


FIG 6 Bone marrow of normal guinea pig × 1,330 Small lymphocytes (L) are scattered throughout the marrow parenchyma, and three are present in this field They are among the smallest cells in the marrow, and the only cells approaching them in size are the smaller nucleated erythroid cells These are easily recognized by their dense and pyknotic nucleus

unpublished data) we have tried to follow the labelling of cells in guinea pig bone marrow. Table II gives preliminary results in

Table II

LABELLING OF MARROW LYMPHOCYTES AND TRANSITIONALS AFTER THYMIDINE

No of experiment	*Hours after thymidine*	*Lymphocytes*		*Transitionals*	
		Counted	*Labelled*	*Counted*	*Labelled*
113	2	200	0	35	20
106	6	200	3	30	8
103	7	200	12	Not counted	
92	24	200	30	—	—

Unpublished data, Everett, Reinhardt and Yoffey (1959)
In the last two experiments many of the transitionals were damaged and others were difficult to identify They have therefore been recorded as "not counted" "Lymphocytes" means small lymphocytes

four animals. The small lymphocytes do not label within the first few hours. However, during this time the transitional cells are labelling actively. From four hours onwards, increasing numbers of labelled small lymphocytes appear in the bone marrow (cf. Schooley, Bryant and Kelly, 1959). Since the transitional lymphocytes undergo mitosis very infrequently, if at all, these labelled small lymphocytes must have entered the marrow from the blood stream. A fuller discussion of the reasons against the view that lymphocytes are being formed in the marrow will be found elsewhere (Yoffey, 1960).

If these observations can be substantiated, they would fit in with the thesis that the small lymphocyte reaches the marrow in an inactive phase, incapable of DNA synthesis and growth, but after a while becomes transformed into the transitional cell (Yoffey, 1957) which is capable of DNA synthesis, and which grows rapidly until it reaches the blast cell stage, when mitosis occurs. Mitoses in the group of transitional cells, as already noted, are singularly infrequent. Further there are countless gradations of size from the small lymphocyte via the transitional lymphocyte to the blast cell.

Migration of lymphocytes to the marrow

The evidence for the migration of cells to the marrow is both direct and indirect. The indirect evidence is twofold: *first*, that the marrow contains large numbers of lymphocytes, with no indication of their being formed there; *second*, that the marrow lymphocyte population may undergo substantial increase in too short a time for this to have been due to multiplication *in situ*.

The direct evidence consists of the identification in the marrow of labelled lymphocytes injected into the blood stream. The experiments of Farr (1951) and Fichtelius (1953) were of this type. Perry and co-workers (1959), in the dog, transfused autologous lymphocytes which had been labelled *in vitro* with ^{32}P, and found appreciable activity in the marrow. Elsewhere in this volume Everett and co-workers (1960) report the results of transfusing into littermates lymph or blood containing labelled lymphocytes from rats which had received injections of tritiated thymidine. These labelled cells were found in spleen, lymph node, Peyer's patches and bone marrow, but not in lung. Ambrus and Ambrus (1959) found that large lymphocytes which had been labelled *in vitro* with thymidine did not recirculate; a few were found in bone marrow and lymph nodes Some of these experiments involve varying degrees of lymphocyte manipulation, and expose the cells to conditions which may be far from physiological.

A preliminary report by Bond (1959) suggests a promising approach. Working with parabiotic rats, he injected thymidine into one animal, while the connexion between the two was clamped for one hour. At the end of this time the clamp was removed and the labelled cells could then pass from the donor to the recipient animal. Labelled mononuclear cells, both large and medium-sized, were found in the bone marrow, spleen and lymph nodes of the recipient. After 24 hours the cells had undergone morphological changes whose significance was not clear.

In our own experiments (Everett, Reinhardt and Yoffey, 1959,

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If the small lymphocyte is being formed in the marrow, and if the transitional cell is an intermediate stage between it and a blast cell precursor, the process must be merely one of gradual nuclear shrinkage with shedding of most of the basophilic cytoplasm, while what little is left gradually loses its basophilia. There also arises the quantitative problem of the small number of the blast cells, which seem to be inadequate for the needs of erythropoiesis and granulopoiesis, without the added burden of lymphocytopoiesis.

One final point of interest emerges from the data of Maloney and Patt (1958). The labelled lymphocytes which they found in the bone marrow were small, whereas neither Cronkite and co-workers (1959) nor Perry and co-workers (1959) appear to have found labelled small lymphocytes in the thoracic duct lymph of the dog. If these cells nevertheless appear in the bone marrow, one may speculate either that the labelled small lymphocytes obtained direct entry into the blood stream, or else that the labelled larger and medium lymphocytes which left the precursor pool completed the final stages of the reduction pathway elsewhere.

Whether this interpretation be correct or not, there is now sufficient evidence from what one may term the more physiological of the labelling experiments to indicate that lymphocytes from the blood stream may find their way in appreciable numbers to the bone marrow, though it has not been possible as yet to estimate them quantitatively, or to determine the fate of the labelled cells.

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DISCUSSION

Cronkite: Anaesthesia is necessary in most of these experiments and it makes a fantastic difference in the output of the thoracic duct lymphocytes in the various species. Are these being counted under identical conditions so that the output per kg. is under the same experimental conditions?

Yoffey: The guinea pigs and rats were under Nembutal, and I believe your dogs were also under Nembutal. I quite agree about the effect of anaesthetic, and we have had that problem throughout with lymphocyte production. Many years ago I anaesthetized a series of animals, did blood counts at intervals and found a fall in the blood lymphocyte count (1935, *loc. cit.*). That of course just aggravates the problem. One has the feeling that throughout one is getting consistently low figures for lymphocyte production Prof. Reinhardt and I (1957, *loc. cit.*), tried in the guinea pig to do a very rapid cannulation—anaesthetize, cut into the duct quickly, tear it and put a capillary tube there. Very often we would be collecting lymph after ten minutes, just by capillary suction. We thought we would reduce stress to a minimum. Whether we did or did not I don't know, but the actual figures for lymphocyte output did not seem to vary from those generally obtained.

Cronkite: I would like to introduce at this stage the fact that for the last two years Dr. Bond at Brookhaven has been carrying on a large series of studies with cross-circulation, parabiosis, and transfusion of tritium-labelled lymphocytes. To date, with lymphocytes labelled or unlabelled, it has not been possible to demonstrate any protection against radiation injury.

Next, a significant percentage of all classes of lymphocytes can eventually be labelled, yet after transfusion, parabiosis or cross-circulation, when the animals are sacrificed at regular intervals and a systematic search is made for the labelled cells, one can admittedly find labelled cells in the thymus, spleen, and lymph nodes, but to date we have found not one labelled erythroid cell, and only a very rare neutrophilic precursor that possibly came in through the thoracic duct—we do not know if there was any transformation The only things, in this type of experiment, that we have found labelled that would begin to

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if the theory were correct—all of them would undergo myeloid metaplasia from the outset. Now in fact they do not, although they do at certain periods of foetal life, and they can do so even during adult life, though they do not do so often. Whenever I felt doubts about the [illegible]

there are a lot of other odd facts about it to explain. If we are right, then I do not know how to explain these other facts to which I have referred.

Lajtha: Regarding lymphocyte transfusion in protection against radiation, I think the main question is: what sort of protection? You can get some sort of protection by transfusing or injecting lots of things into animals, but that is rather different from the type of protection which you get when you transfuse intact myeloid elements Stuart Finch and his group in New Haven have shown (personal communication) that even a completely cell-free RNA extract can protect to some degree against certain doses of radiation. When it comes to proving whether it is cellular protection, or some humoral factor (which may be a very real adjuvant), then I think the type of experiment which Dr. Loutit has done is very useful. In these experiments you irradiate your control suspension with a dose which you know leaves no viable cells, and if the protection is still significant then you can say that the protection is due to a humoral factor and not to cell colonization.

Prof. Yoffey, you mentioned that rats and small rodents, because of [illegible]

smaller or a very much larger number of cells simply by turning over slower or faster. The question is . what proportions of the existing stem cell compartment are in active turnover?

Yoffey: [illegible]

cells are differentiating?

Lajtha: I suggest that as a possibility.

suggest—but certainly not prove—any transformation of cells are labelled plasma cells. Perhaps this is not a good experiment. We have also done this sort of thing in the dog and in the guinea pig, in experiments in which the circulation to the lower part of the body was occluded and the lower extremities kept viable by extracorporeal circulation of compatible blood. The labelling procedure takes place in the upper part of the body for a period of time adequate to metabolize or incorporate all of the administered thymidine. Then the circulation is re-established, serial bone marrows are taken, and again we find no labelled erythroid precursors, no labelled neutrophilic precursors, and only an occasional plasma cell, large lymphocyte and reticulum cell that is labelled

Yoffey: I thought Bond's work (1959, *loc. cit*) was very intriguing. He reported finding labelled mononuclear cells in the marrow. I had meant to refer to that, but was leaving it to Dr. Everett to present data on the finding of some labelled cells in the marrow and whether they [illegible]

[illegible]

which your findings would be a conclusive answer: by the time you have got this label into your cell does it function as a normal cell? I do not know. All I can say is that if those cells are normal cells, and if they never develop any further, then lymphocytes would apparently not have those potentialities. There still remain many problems.

Lymphocyte transfusion to give protection is another matter again The literature is not very extensive, but certainly the consensus of opinion is that it does not protect In an early paper (Congdon, Uphoff and Lorenz, 1952, *loc. cit.*), however, it was reported that in a few cases thymocyte suspensions seemed to protect, but this was never pursued any further. Recently Salvidio, Oliva and Pierotti (1958, *loc. cit.*) gave calf lymph node to rats and claimed to get protection; they said that other people had not got protection because they had not given big enough amounts. I am not a transfusion expert, but on the basis of the story as we see it unfolding with thymidine, something has got to activate the lymphocyte, and apparently it does not get activated in lymphoid tissue. If it did you would never have lymphoid tissues,

lymphocyte, what you notice as the cell enlarges is that the cytoplasm gradually becomes more basophilic. That is not a unique thing; you get it in the lymph node too when you have what everybody accepts as a stem cell, the so-called primitive reticulum cell with a non-basophilic cytoplasm, which gradually develops basophilia and becomes the lymphoblast.

Lamerton: Labelled cytidine studies might show up something important, and might answer Prof. Jacobson's question as to which of the cells are transforming, if in fact they are transforming. I think the emphasis has been so much on DNA that we have forgotten what we could learn by looking at the RNA.

Yoffey: I think that is very sound, and I only wish you or someone else would do these labelled cytidine studies.

Everett: Dr. Rieke in my laboratory has cultured lymphocytes, labelling them with cytidine and some of the other precursors that would go into the RNA, and he does indeed find that there is a relatively high turnover in the RNA compound but not in the DNA.

Braunsteiner: In a recent experiment (not yet published) we spleen-shielded mice by classical methods, and then examined the blood cells hour by hour. Since it was admitted that cells might leave the spleen of these mice and probably go into the bone marrow, we ought to find the prospective stem cell in the blood of these mice before the bone marrow recovers. After three or four days the count goes down to several hundred cells; then between the fifth and eighth days, in our experiments, we find about 60 or 70 per cent of what are called medium-sized lymphocytes with a small but clearly visible nucleolus, and only a very small proportion of small lymphocytes. Shortly before the bone marrow recovers there might be several thousand of these lymphocytes in the peripheral blood. Between the sixth and eighth days we find a tremendous increase, in the bone marrow, of what Prof. Yoffey calls transitional cells, and one or two days later we find normal myelopoiesis. In the peripheral blood we never found any cell which could from a haematological standpoint be called a myeloblast or any classical myeloid precursor—we only found medium-sized lymphocytes. This naturally is very much in favour of what Prof. Yoffey told us.

Yoffey: Have you any experimental evidence?

Lajtha: No.

Yoffey: I think that makes it all the more important to try and define the stem cell. Have you any evidence at all bearing on the stem cell? We have looked at the sinusoidal endothelium very carefully, and have not been able to find any evidence of transformation at all. That was one of the more favoured stem cells in the literature. We have also looked at reticulum cells. We were up against the difficulty that in the smear preparations on which we did our quantitative work, reticulum cells were readily damaged. But in our sections, when we get stem cell regeneration following irradiation (there is a new set of experiments with partial shielding now in progress), then we look at reticulum cells also as a possible source for new formation of haemocytoblasts. That is the stem cell that everybody accepts: as far back as the haemocytoblast stage everybody is happy; it is when you get past the haemocytoblast stage that doubts begin to arise. But in these sections we found all possible transitions in cell size between the small cells and the haemocytoblasts. I can show you whole areas in which there are any number of those transitions, but no more than a very occasional mitosis of a reticulum cell, though I must admit that, largely because of difficulties of identification, we have not performed counts of reticulum cells in sections.

Jacobson. Has anyone tried to differentiate between those lymphocytes which circulate and the tissue which in fact bears them? For example, has anyone studied the effect of transplantation of the mesenteric node, let us say to the bone marrow, in comparison with the circulation of the cells that are leaving? It seems to me this might be important, and the possibility that there might in fact be two populations has, I think, been suggested by someone in Sweden some years ago.

Lamerton: If the small lymphocyte does transform, it must have a very high rate of protein synthesis at some point. Is there any evidence with RNA labelling that this is happening to any small lymphocytes?

Yoffey: We have not done any RNA labelling. The small lymphocyte, when it forms in the lymph node, comes from heavily basophilic precursors which gradually lose their basophilia. In the marrow, in the series of cells which we have interpreted as reactivation of the small

RADIOISOTOPES IN THE STUDY OF BLOOD CELL FORMATION WITH SPECIAL REFERENCE TO LYMPHOCYTOPOIESIS*

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IT has been stated that the research tool provided by the radioactive tracer elements is as fundamental as was the invention of the microscope (Warren, 1949) It is doubtful that this is the case with respect to problems of blood cell formation and development. Nevertheless, it is unquestionable that the application of radioisotopic methods has contributed significantly to our knowledge of haemopoiesis. Furthermore, a continued exploitation of these methods can be expected to play an important rôle in providing answers to many of the unsolved problems we face concerning the origin, function, lifespan and fate of certain of the blood cells.

The utility of a radioactive isotope is due to its nuclear instability which provides a sensitive means for following its uptake and fate It has been estimated that the sensitivity of the radioisotopic methods is of the order of 10^8 times greater than the most sensitive gravimetric methods. It is to be borne in mind, however, that the radiation emission associated with nuclear instability can disturb the equilibrium of normal cell processes. Our assumption that radioisotopes (in doses commonly employed for blood cell

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We are labelling the spleen cells of these animals by injecting thymidine shortly after irradiation and spleen-shielding. After a short time labelled cells can be found in the peripheral blood and they settle down in the bone marrow. By this method we hope that it will be possible to determine the nature of these motile cells from the spleen and to follow step by step their fate in the bone marrow. Quantitative data will be presented soon.

superiority of radioisotopic methods over most other methods of cell labelling.

Radioisotopic techniques employing conventional counter methods have been successfully applied in studies of leucopoiesis as well as in studies of erythropoiesis. These methods simplify the quantitative approach to the problem. The methods are often limited, however, by the need for obtaining relatively large samples of one blood cell type; this is difficult to achieve except for red blood cells. Thus, the most successful applications of these counter methods have concerned the erythrocyte which has been labelled with a variety of radioisotopes including ^{55}Fe, ^{59}Fe, ^{32}P, ^{14}C, ^{35}S, and ^{51}Cr (see review by Berlin, Waldmann and Weissman, 1959).

Radioautographic techniques applied to the study of blood cell production have been most rewarding. They provide for the precise identification of labelled cell types. Thus, it has been possible by employing radioautography with selective radiomaterials to learn much about the site of origin, rate of production, lifespan and fate of the individual leucocyte cell types within mixed populations of cells (Patt, 1957; Bryant and Kelly, 1958; Bond *et al.*, 1959; Cronkite *et al.*, 1959; Diderholm and Fichtelius, 1959; Lajtha, 1959*a*, *b*; Patt and Maloney, 1959; Schooley, Bryant and Kelly, 1959; Yoffey, Everett and Reinhardt, 1959; and Everett, Reinhardt and Yoffey, 1960). Radioautography has also been applied effectively in determining the generation time and lifespan of the erythrocytes (Lajtha and Suit, 1955; Harriss, 1957; Everett and Yoffey, 1959; and Lamerton, Belcher and Harriss, 1959).

Radioautography combines the advantages of the classical histological methods with those of the radioisotope. The theory, practical applications, limitations and shortcomings of the method will not be presented here since they have been adequately discussed elsewhere (Gross *et al*, 1951). It is perhaps desirable, however, to present certain technical aspects of radioautographic

labelling) produce no significant radiation damage to the cells may not be valid in all instances. For example, Painter, Drew and Hughes (1958) have shown an inhibition of HeLa cell cultures grown on a medium containing 2·5 to 5 μc/ml. of [^{3}H]thymidine. Taylor (1958) noted chromosomal aberrations within *Bellevalia* containing this same concentration of [^{3}H]thymidine. Drew and Painter (1959) observed that very few HeLa cells survive and continue to divide after growing in a medium containing [^{3}H]-thymidine at concentrations of 0·1 μc/ml. or greater. Wimber (1959) has observed fragmentation of chromosomes in the root tips of *Tradescantia paludosa* grown in 1 or 2 μc/ml. of [^{3}H]thymidine. The mitotic index dropped sharply in roots which had been exposed to 1 μc/ml. of [^{3}H]thymidine for periods of more than eight hours. It is gratifying, however, that with *in vivo* studies Cronkite and co-workers (1959) found no evidence of classic cytological effects of radiation in mammalian cells with doses up to 2 μc/g. of [^{3}H]thymidine. This dosage is of the order of two or more times that required to produce effective labelling of cells in haemopoietic and other proliferating tissues Johnson and Cronkite (1959) reported that the biological effects of [^{3}H]thymidine in doses under 1 μc/g. body weight on mouse spermatogonia are comparable to those of less than 2 r. of γ-irradiation per day.

An additional consideration which is perhaps of lesser import than that of radiation damage is the possibility that the decay product of radioactive elements used for labelling may be toxic to the cell. Finally, an assumption we make in employing radioisotopes is that the radioelement has the same chemical reaction within the cell as does the normally occurring element. This may not be correct in all instances, particularly when the difference in mass between two isotopes is considerable. To our knowledge, however, this has never been reported as a limitation. The fact that a radioelement can be expected to exhibit the same chemical reactions as the normally occurring stable form accounts for the

provides for a thin uniform layer, lessens the chance of emulsion slippage during subsequent processing and generally insures against formation of artifacts.

In the case of tissue and blood smears, however, the nitrocellulose covering impairs the staining reaction. Thus, we now process smear preparations of haemopoietic tissues without coating with nitrocellulose. The conventional blood stains cannot be used prior to covering the tissues with photographic emulsion since they contain methylene blue or other similar dyes which are reducing agents and thus effect a histochemical reduction of the emulsion.

The procedure we now employ is to dip the glass slides in a weak solution of gelatin (0·5 g. gelatin and 0·05 g. of chrome alum in 100 ml. of H_2O) before making the tissue smears. The smears are then fixed in absolute methanol, dried and coated with melted Eastman Kodak NTB3 emulsion diluted in the ratio of two parts of emulsion to one part of water. To each ml. of this dilution, two drops of a 1 per cent solution of the detergent Dupanol (Proctor & Gamble Co., Cincinnati) are added. This solution in combination with the gelatin-covered slides promotes an even spreading and adherence of the emulsion. The slides, after drying, are then placed in light-tight boxes containing a desiccant and exposed for two to three weeks at 5°. After development in the conventional way, the preparations are stained for 2 to 15 minutes in a 1 per cent solution of Leishman–Giemsa of pH 6·7 at 5°. Differential de-staining is done with H_2O; after drying in air coverslips are applied using a neutral mounting medium.

The only real difficulty we have encountered with this procedure has been the occasional histochemical reduction produced by smears of thymus and less frequently by bone marrow. This difficulty can be surmounted, however, by subjecting the smears to several changes of H_2O after initial fixation. It was previously demonstrated (Everett and Simmons, 1953) that water is most

applications to studies of blood cell formation when using tritium-labelled compounds.

Tritium is most satisfactory for radioautography, as was shown initially by Fitzgerald and co-workers (1951), because the low energy of its beta particle (0·018 mev) gives high resolution and provides for discrete localization. Thymidine was shown to be a specific precursor of deoxyribonucleic acid (DNA) (Reichard and Estborn, 1951). Thus, with the development of [^{3}H]thymidine (TTH) by Hughes (cited by Wimber, 1959) and by Verly, Firket and Hunebelle (1958), an excellent tool became available for studies of cell formation, lifespan and fate. This has been demonstrated by a number of recent studies from the Brookhaven Laboratories (Bond *et al.*, 1959; and Cronkite *et al.*, 1959) as well as by others. Cronkite and co-workers (1959) have presented a good account of the premises made in using TTH for studies of DNA synthesis and cell turnover in haemopoietic tissues. Furthermore, the use of TTH in studies of blood cell formation is to be discussed by Dr. Cronkite at this symposium. Thus, a specific consideration and critique of the TTH technique in relation to our studies will not be made.

Radioautography using tritium-labelled compounds

In our experience, the integrated method of radioautography developed by Belanger and Leblond (1946) has been the method of choice. The only significant change which we have made in the method for the localization of tritium in sections of haemopoietic tissues is to dip the mounted sections twice instead of three times in a 1 per cent solution of nitrocellulose in ether-alcohol. With this method, we have obtained most satisfactory silver reduction and discrete localization of tritium in smears having a protective layer of nitrocellulose less than 0·5 μ in thickness (Everett, Reinhardt and Yoffey, 1960). The nitrocellulose film promotes the spreading of the emulsion over the tissues,

into small pieces and teasing in homologous serum. Sections were cut at 5 μ of thymus, bone marrow, mesenteric lymph node, spleen, Peyer's patch and cervical lymph node after fixation in Bouin's fluid and embedding in paraffin.

The mounted tissue sections were stained in haemalum for two hours and in 1 per cent eosin in 70 per cent alcohol for 5 minutes. Radioautographs of the sections and smears were prepared as described earlier (Everett, Reinhardt and Yoffey, 1960) except for the slight modifications indicated above for the tissue smears. Two weeks was the standard period of exposure for all slides and, in most instances, additional slides from each experiment were exposed longer.

The difficulty of placing thoracic duct lymphocytes in the three conventional categories, large, medium and small, upon the basis of absolute measurements in smear preparations was considered previously (Everett, Reinhardt and Yoffey, 1960). This difficulty of classifying the cells upon the basis of size is equally applicable to smears of blood and of the minced tissues. In our recent studies in the rat, however, we have employed cell measurements in classifying lymphocytes in all smear preparations; this provided a better basis for comparing the counts made by different individuals. Lymphocytes of 7 μ or less in diameter are considered small, those more than 7 and less than 10 μ as medium, and cells 10 μ or more are classified as large.* These limits were chosen on the basis of a frequency distribution chart prepared by measuring the diameter of many lymphocytes in several different smears. It may be said that the lymphocytes having a diameter of less than 7 μ possessed the characteristic high nucleus to cytoplasm ratio and polar cytoplasm.

As reported previously (Everett, Reinhardt and Yoffey, 1960), labelled large and medium-sized lymphocytes appeared in thoracic duct lymph of the guinea pig within the first hour after TTH administration. Labelled small lymphocytes began to appear in

* The respective lymphocytes of the guinea pig are slightly larger.

effective in removing histochemical reducing agents from fixed tissues.

Tritium labelling and the production of lymphocytes

Labelling with single injections

We have employed the radioautographic procedures described above in studies of lymphocyte production, lifespan and fate in the guinea pig and rat. Since our observations relative to the appearance of labelled cells in the thoracic duct lymph of the guinea pig have recently been published (Yoffey, Everett and Reinhardt, 1958, 1959; and Everett, Reinhardt and Yoffey, 1960), we propose to give major consideration to more recent studies using the rat, and to present the results of our efforts to answer certain problems of lymphocytopoiesis raised in the earlier studies.

The animals used included male rats of the Sprague-Dawley strain weighing 200–250 g., and male guinea pigs of a mixed strain weighing about 400 g. Tritiated thymidine (Schwarz), having a specific activity of either 0·36 or 1·9 c/m-mole*, was given intraperitoneally or intravenously to the guinea pigs, and intraperitoneally to the rats, in doses of 1 μc/g. body weight. At varying periods, thoracic duct lymph was collected as described by Reinhardt (1945) and Reinhardt and Li (1945) for the rat and by Reinhardt and Yoffey (1957) for the guinea pig. Smears were made of lymph which had been concentrated in serum as described previously (Yoffey and Courtice, 1956; and Everett, Reinhardt and Yoffey, 1960). Blood films were made at times coinciding with the periods of lymph sampling. At the termination of the experiment, smears were made of bone marrow suspensions obtained by a technique previously described (Yoffey, 1955). Smears were also made of thymus and of superior mesenteric lymph node suspensions prepared by cutting these tissues

* With few exceptions, guinea pigs received the 1·9 c/m-mole [^{3}H]thymidine and rats the 0·36 c/m-mole.

Figs. 3–6 are representative radioautographs of labelled lymphocytes in rat thoracic duct lymph at different times after TTH administration. It was observed, as reported previously (Everett, Reinhardt and Yoffey, 1960), that the cells of the early intervals are heavily labelled and that the intensity of labelling is decreased in cells of the later intervals. This decrease in intensity of labelling is apparent for the small lymphocyte as well as for the large and medium-sized cells.

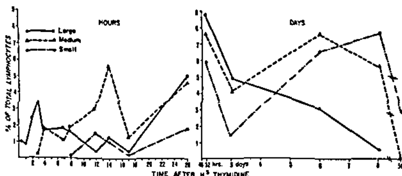


Fig. 2. Labelled lymphocytes in blood of guinea pigs.

The lymphopoietic centres (spleen, thymus, lymph node and Peyer's patch) of the rat as well as of the guinea pig showed abundant proliferative activity (Fig 7). The extensive labelling in these centres made it evident that there was a large reservoir of newly formed lymphocytes which could be released to thoracic duct lymph

Labelling with multiple injections

The data presented in Fig. 1 show the maximum percentage of labelled lymphocytes in thoracic duct lymph of the guinea pig after a single dose of TTH to be 6 to 7 per cent, of which about half, or 3 per cent, are small. Furthermore, the percentage of

the fourth hour and increased in numbers as the proportion of labelled large and medium lymphocytes decreased. This sequential appearance and the percentages in which the labelled large, medium and small lymphocytes appeared in thoracic duct lymph are shown graphically (Fig. 1).

The percentage distribution of labelled lymphocytes in blood with time follows the same pattern as that of lymph (Fig. 2).

This pattern is also typical for the labelled lymphocytes in the

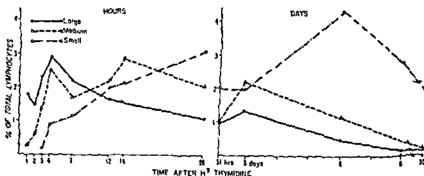
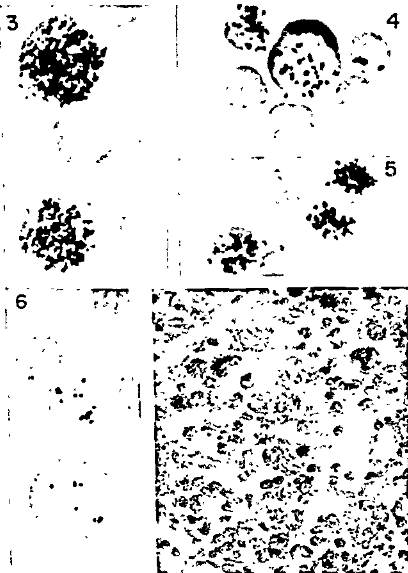


Fig. 1 Labelled lymphocytes in thoracic duct lymph of guinea pigs.

smear preparations of lymph nodes and thymus at the corresponding time intervals.

The above observations were repeated in the rat at the same intervals after TTH administration. Although a full account of these observations for the rat is not included here, it may be said that the results were similar to those of the guinea pig studies. The only essential difference was a lower percentage of labelling for the rat thoracic duct and blood lymphocytes. For example, the thoracic duct lymph of the rat at 6 to 7 hours after a single dose of TTH contained 4 per cent of labelled cells, the highest percentage observed. In comparison, 5·4 per cent of the cells in guinea pig lymph were labelled at this time.



Figs 3–6 are radioautographs of lymphocytes from thoracic duct lymph of rats subsequent to the administration of *1 μc/g of tritiated thymidine*

Fig 3 Labelled large and medium lymphocytes 0–2 hrs after TTH, 14 days exposure (× 1,800)

Fig 4 Labelled small and large-medium lymphocytes 4½–5½ hrs after TTH, 21 days exposure (× 1,800)

Fig 5 Labelled dividing lymphocyte 4½–5½ hrs after TTH (× 1,800)

Fig 6 Weakly labelled lymphocytes, small and large, 72 hrs after TTH, 21 days exposure (× 1,800)

Fig 7 Lymph node showing numerous labelled cells 2 hrs. after TTH, 56 days exposure (× 258)

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labelled lymphocytes in blood is approximately the same for most of the corresponding intervals. It should be noted, however, that the labelling intensity of the majority of small lymphocytes appearing in lymph or blood three or more days after thymidine administration is much reduced (Fig. 6); this suggests that these small lymphocytes arose from lightly labelled larger precursors present at these later intervals. We have no evidence as to the fate of the heavily labelled small lymphocyte; its lifespan in the blood is brief.

It seemed possible that the failure to label a greater percentage of the small lymphocytes with a single dose of TTH might be due to a very short intermitotic period for the precursor cells. This would imply a proportionately brief DNA synthetic period and would mean that a much smaller percentage of the daily output of lymphocytes would be labelled from a single injection of TTH. In order to test this possibility, rats were given multiple doses (1 μc/g.) of TTH as follows: two received three doses each at two-hour intervals, two received three doses at five-hour intervals; one received six doses at 30-minute intervals. Twenty-four hours after the last injection, thoracic duct lymph was collected and radioautographs were prepared as described above.

The percentage of labelled cells was increased only slightly in these animals over that in lymph collected 24 hours after a single dose of thymidine. In no instance was the increase more than 1 to 2 per cent

As a sequel to these experiments, it seemed appropriate to go to the other extreme and to administer multiple doses of TTH at longer intervals. For this, a male rat was given an injection dose of 1 μc/g. at weekly intervals for six weeks. Thoracic duct lymph was collected one week after the last injection. Radioautographs of this lymph, after the standard two weeks' exposure, revealed that 8·6 per cent of the lymphocytes were labelled, most of these being small. The labelling for the majority of the cells was weak, as in the previous instances at longer intervals following

thymidine administration. It is probably significant, however, that with prolonged exposure periods, considerably more small lymphocytes from this animal effected a reduction* of the emulsion sufficiently above the background level to be interpreted as labelled cells. After 20 days' exposure, 16 per cent of the cells were classified as labelled and after 62 days, 30 per cent were so characterized. That more lymphocytes registered radioauto- [illegible]

radioautographs of lymph from a rat one month after TTH evidenced 1·6 per cent labelled lymphocytes after 14 days' exposure and 2·6 per cent after 56 days' exposure. In contrast, lymph samples collected 24 hours after injection of TTH in each of two cases showed essentially the same percentages of labelled lymphocytes after exposure periods of 9, 14, 27, 42 and 56 days. This has been the case, too, for lymph collected at still shorter intervals and exposed for 14 and 21 days. It is readily apparent that these observations signify the need for a most careful consideration in evaluating labelling intensity and percentage labelling with respect to exposure time and with respect to the interval of sampling. Two weeks' exposure was more than adequate for the

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FIGS. 8–10. Labelled medium and large lymphocytes from teased preparation of thymus (Fig. 8) and of lymph node (Fig. 9) after 20 minutes *in vitro* culture in TTH; 14 days exposure (× 1,800.)

FIG. 10. [illegible]

FIG. 11. [illegible]

FIGS 12 and 12A Labelled reticular cells and non-labelled lymphocytes of lymph node from guinea pig with pertussis-induced lymphocytosis 24 hrs after TTH. The reticular cell at the upper left of Fig. 12 was focused to show the cytoplasm. The lymphocytes are dense due to the longer staining necessary to show the reticular cells in better detail. Fourteen days exposure. (× 1,800)

* Two or more grains per cell as opposed to a background of less than one grain per unit area comparable to that of the cell.

8



10



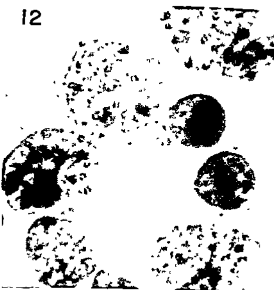
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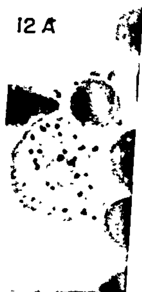
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12



12 A



20 minutes at 37°, and then centrifuged, washed and suspended as above. In all cases, approximately 0·15 ml. of serum-suspended lymphocytes (total cells = about 9×10^6) was then placed in the diffusion chambers which previously had been moistened with heparinized Ringer's solution.

The diffusion chambers, slightly modified from those of Algire, Borders and Evans (1958), were made from Lucite rings of $\frac{3}{16}$" thickness, 0·5 inch outside and 0·25 inch inside diameter. Diffusion membranes 30 μ thick with average pore diameter of 0·45 μ were secured to these rings with Millipore cement, × × 70 000 00.

The lymphocyte-bearing chambers were then placed through ventral midline incisions for culture in the peritoneal cavities of rats selected from male littermates of the donor animals. Chambers were cultured for one to three days; smears of chamber contents as well as imprints of diffusion membranes were then prepared for radioautographic processing. Control smears of lymph, made immediately after *in vivo* or *in vitro* labelling, were radioautographed at the same time.

In addition to these experiments with cultured thoracic duct lymphocytes of the rat, cell suspensions were made of the thymus and of the cervical lymph node of a guinea pig and labelled *in vitro* as described above for the lymph (Figs. 8 and 9). Cells from each suspension were placed in separate Millipore chambers and introduced into the abdominal cavity of the same animal. After 24 hours, smear preparations were made and radioautographed along with the control slides. A labelled dividing cell from one of these preparations is shown in Fig. 10.

At the present time, results are available for only four experiments in which thoracic duct lymphocytes were cultured. The observations, similar to those of Schooley and Berman (1960), seem unequivocal, however, in showing the small lymphocyte and thymocyte to be derived from their medium-sized counterparts, which in turn are derived from larger precursors. In all experiments, control radioautographs showed that the only

labelled cells of the early intervals and with longer exposure many of the more heavily labelled cells could not be classified because of the intense reduction of the overlying emulsion.

In vivo culture of labelled lymphocytes

For the following reasons, the results from both the guinea pig and rat studies indicate the developmental sequence of the lymphocyte to be large→medium→small. (1) labelled large and medium-sized lymphocytes are present in all tissues at the earliest intervals studied, although most are large; (2) the proportion of labelled medium cells increased during the first few hours; (3) labelled small lymphocytes first appeared about the fourth hour after thymidine administration; and (4) the average intensity of labelling decreased from large to medium to small lymphocytes. *The possibility still existed, however, that the labelled* small lymphocyte arose from other labelled precursors whose identity was overlooked. In an attempt to provide a definitive answer to this question, it seemed appropriate to culture labelled lymphocytes in Millipore chambers to allow direct determination of changes in size distribution of labelled cells.

For these experiments, lymphocytes were labelled either *in vivo* or *in vitro*. Lymph cells labelled *in vivo* were collected from the thoracic ducts of adult male Sprague-Dawley rats which had received parenterally $1\ \mu c/g$ body weight of TTH two hours previously. Lymphocytes for *in vitro* labelling were obtained from thoracic duct lymph of uninjected animals. Lymph was collected for 30 minutes to one hour in a small vial in which minute quantities of powdered heparin and 400 units of potassium penicillin-G had been placed.

cyte production, thymidine has been given to animals having a sustained lymphocytosis. A lymphocytosis was induced in two guinea pigs and in one rat by injecting suspensions of killed pertussis bacilli as described by Tuta (1937) for the rabbit. The injections of from 1 to 2×10^9 bacilli each were given at two to three-day intervals until the peripheral blood counts evidenced a sustained lymphocytosis. One guinea pig with an initial leucocyte count of 4,600 per cubic millimetre (65 per cent lymphocytes) showed an increase to 18,860 (85 per cent lymphocytes) in eight days. The leucocyte count of the other animal increased from approximately the same initial value to 13,340 (85 per cent lymphocytes) in two weeks. The leucocyte count of the rat increased from 15,700 to 22,300 in two weeks with no change from the initial 74 per cent lymphocytes. At these respective times after the onset of the pertussis injections, each animal was injected with 1 µc/g. of TTH. After 24 and 48 hours respectively, samples of thoracic duct lymph, blood, lymph node and thymus were obtained and processed for radioautography as described above. The percentages of the respective labelled cell types in thoracic duct lymph and in the glandula mesenterica magna appear in Table II.

For comparison, the percentages of labelled cells in control animals are given for the corresponding periods after thymidine administration. It is observed that the labelled cells increased in each case and range from about 50 to 150 per cent above the control values. This increase is of the same order of magnitude as might be expected from the increase in the lymphocyte content of blood.

It may be said that there was also a significant increase in the percentage of labelled cells in the lymph nodes and thymus. Of special interest is the increased number of reticular cells as well as large numbers of [illegible] cells, many of which are labelled.

labelled cells introduced into the Millipore chambers were of the large and medium varieties. After culturing for 24 hours or more, the labelled cell types were predominantly medium and small (Fig. 11). The data for thymus and lymph node cells are presented in Table I.

It is perhaps significant that the percentage of cells labelled from *in vitro* cultures of thymus was more than twice the percentage labelled in lymph node. Schooley, Bryant and Kelly (1959) similarly reported a greater percentage labelling of thymocytes

Table I

CHANGES IN SIZE DISTRIBUTION OF CELLS LABELLED *in vitro* AND CULTURED *in vivo* IN DIFFUSION CHAMBERS

In vitro CONTROL:

Tissue	Cells counted	% Cells labelled	Labelled cell types as % of total cells		
			L	M	S
Lymph node	743	2·8	0·8	1·9	0·00
Thymus	605	6·8	0·4	6·3	0·00

In vivo 24-HOUR CULTURE:

Tissue	Cells counted	% Cells labelled	Labelled cell types as % of total cells		
			L	M	S
Lymph node	673	3·1	0	2·1	1·0
Thymus	626	7·5	0	4·3	3·2

L—Large, M—Medium, S—Small

than of lymphocytes after *in vitro* culture of rat thymus and lymph node suspensions in TTH. These observations are also in accord with the studies of Andreasen and Ottesen (1945) which showed a greater renewal of DNA for thymus than for lymphoid organs.

Lymphocyte labelling in pertussis vaccine-induced lymphocytosis

In attempts to answer the question of whether our procedures employing TTH provide for an accurate assessment of lympho-

one, approximately half of the labelled cells are damaged. Although most of these cells could not be classified as to cell type, it is our impression that by far the largest numbers of damaged cells were reticular in type.

Recirculation of transfused labelled lymphocytes

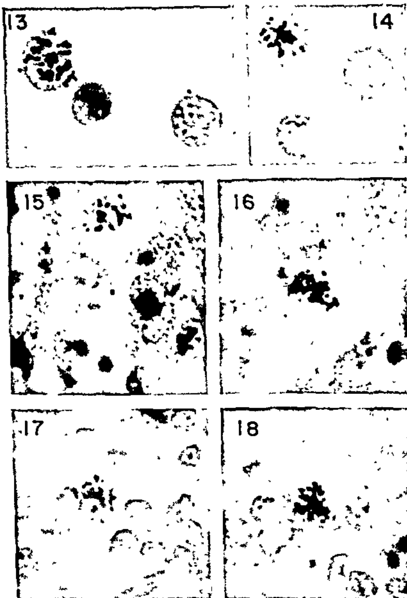
Reference was made above to the fact that only a small proportion of lymphocytes appearing in thoracic duct lymph after a single dose of TTH were labelled. Assuming the premises of TTH labelling to be correct, this shows that only a small percentage of the lymphocytes in thoracic duct lymph are newly formed. This is in accord with the studies of Gowans (1959) who recovered at least 80 per cent of intravenously administered lymphocytes from the thoracic duct. The experiments of Gowans (1959) on infusing unlabelled or ^{32}P-labelled cells provide very suggestive evidence of extensive recirculation. Yoffey and Drinker (1939) reported recirculation but felt it to be on a small scale. The critical test of recirculation is thought to reside in the direct identification of recirculating cells. For this purpose TTH labelling is considered to be more specific in the sense of providing for the actual identification of labelled recirculating cells. With the hope of contributing more direct information relative to recirculation, attempts have been made to recover tritium-labelled lymphocytes in blood, lymph and tissues subsequent to intravenous administration. Male rats of the Sprague-Dawley strain have been used and labelling was effected using our standard dose of 1 μc TTH per gram body weight. Thoracic duct lymph (0·5 to 1 ml.) was collected in separate iced vials from each of five animals. This was given by vein to littermates of the donors. The number of lymphocytes administered varied from $1{\cdot}0 \times 10^7$ to $8{\cdot}1 \times 10^7$, of which approximately 3 per cent were labelled as determined by control radioautographs. Thoracic duct lymph was collected from the recipients after 20 to 30 *minutes* and after 4, 5 and 18 hours. Radioautographs were prepared of the lymph, blood, and

Table II

LABELLED LYMPHOCYTES IN ANIMALS WITH PERTUSSIS-INDUCED LYMPHOCYTOSIS COMPARED WITH CONTROLS
(cell types as % of total cells counted)

	Guinea pig 24 hrs after TTH					*Guinea pig* 48 hrs after TTH					*Rat* 24 hrs. after TTH				
	Total	L	M	S	D&R	Total	L	M	S	D&R	Total	L	M	S	D&R
Thoracic duct lymph															
Expt.	9 6	1 6	3 7	4 3		10 1	2 2	4·7	3·3		6·5	1·7	2·8	1·2	0·9
Controls	6 3	1 1	2 1	3·1		4·1	1 0	1·0	2 0		3·1	1·3	1·2	0·6	
Glandula mesenterica magna															
Expt	13 5	1 9	2 3	3·0	6·4	18·4	0 9	6 0	2·0	9·5	10·1	1·5	3·4	1·9	3·3
Controls	6 7	0 22	1·5	3·7	1·1	11 7	0 02	1·5	9·7	0·41	4·2	0·9	1·1	0·9	1·2

L—Large, M—Medium, S—Small
D & R—Damaged and reticular cells.



FIGS 13–18 Labelled ...

FIGS ... thoracic duct lymph, 28 days exposure (× 1,800)

FIGS 15 and 16 Labelled lymphocytes in bone marrow, 21 and 14 days exposure respectively (× 1,800)

FIG 17 Labelled small lymphocyte in lymph node, 21 days exposure (× 1,800)

FIG 18 Labelled medium-large lymphocyte in spleen, 14 days exposure. (× 1,800)

facing page 60

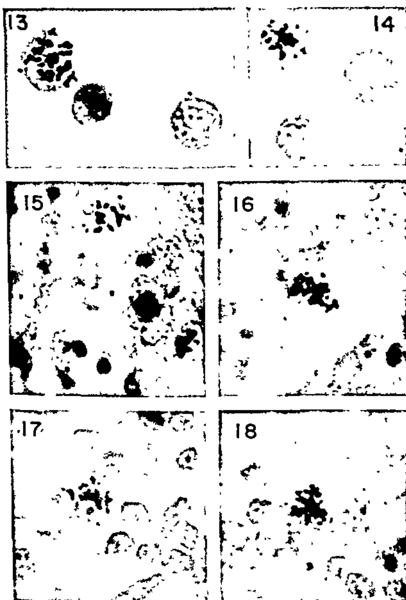
of suspensions of the haemopoietic organs. Labelled small lymphocytes were not observed in the 20- and 30-minute samples of thoracic duct lymph, but a few were found in the 4- and 18-hour samples.

In another series of rats, blood was removed in two to three stages by cardiac puncture and replaced intravenously immediately thereafter with the same volumes of blood from littermates which had received TTH 24 hours previously. The animals were lightly anaesthetized with ether for the procedures; the donors were given 37 μg. of heparin per 100 grams body weight intravenously. These exchange transfusions have been made for five pairs of rats in the weight range of 200–250 grams, and the volumes of blood exchanged have varied from 5·1 to 9·6 millilitres. The number of transfused labelled lymphocytes in the circulation of the recipients was estimated to be from $2{\cdot}3\times 10^6$ to $5{\cdot}2\times 10^6$.

At 6, 12, 24, 36, and 48 hours after transfusions, smears were made of thoracic duct lymph and blood, and in some cases smears were made of bone marrow, thymus, and lymph node. Tissue sections were made of the superior mesenteric and cervical lymph nodes, thymus, bone marrow, spleen and lung. Radioautographs were prepared of the smears and sections as above.

A number of labelled small and occasionally medium-sized lymphocytes were seen in thoracic duct lymph subsequent to the intravenous administration of blood (Figs. 13 and 14). The number of labelled cells observed, however, was small, and they were not seen in all samples from this series. They appeared with greatest frequency in the 24-hour sample. No labelled lymphocytes were observed in the blood 48 hours after administration.

Labelled small, medium and large lymphocytes were also observed in sections of spleen, lymph node, bone marrow and in Peyer's patches (Figs. 15–18), but not in lung. In some sections they were clearly within the capillaries. In other instances, however (lymph node, bone marrow and Peyer's patch), labelled



FIGS 13–18 Labelled lymphocytes in various tissues of a rat 24 hrs after being transfused with blood from a littermate previously injected with TTH

FIGS 13 and 14 Labelled medium and small lymphocytes respectively from thoracic duct lymph, 28 days exposure (× 1,800)

FIGS 15 and 16 Labelled lymphocytes in bone marrow, 21 and 14 days exposure respectively (× 1,800).

FIG 17 Labelled small lymphocyte in lymph node, 21 days exposure (× 1,800)

FIG 18. Labelled medium-large lymphocyte in spleen, 14 days exposure. (× 1,800)

cells appeared among the other cell types within the parenchyma. It may be significant that in proportion to the areas of tissue sections examined, labelled lymphocytes were seen with greatest frequency in bone marrow. This could be related to the vascularity of the tissue. These data show that small and medium lymphocytes pass from blood into lymph and tissues, but do not provide, however, a basis for estimating the percentages of labelled lymphocytes leaving the circulation to reappear in the respective tissues or thoracic duct lymph.

Interpretation and significance of the observations

The sequence in which the labelled large, medium and small lymphocytes appear following a single dose of TTH, the change in their percentage distribution with time, and the results of the *in vivo* culturing of labelled large and medium lymphocytes seem to show rather conclusively that the developmental pattern of lymphocytes is large→medium→small. In addition, the increase in the number of labelled reticular cells and the increase in labelled lymphocytes of the respective sizes following pertussis-induced lymphocytosis are in accord with the "stem cell renewal theory" of lymphocyte formation proposed by Sainte-Marie and Leblond (1958) for the thymus. This theory states that large lymphocytes are derived from reticular cells; the large lymphocytes then pass [illegible]

reticular cell or from a first generation large lymphocyte. Although our data are not adequate to determine the number of generations from stem cell to definitive small lymphocyte, it is apparent from the decreasing grain count that a number of divisions intervene.

Reference has been made to the active cell proliferation within the lymphopoietic centres of the rat and guinea pig as evidenced

by thymidine uptake. It is difficult to reconcile this observation with the relatively low percentages of labelled small lymphocytes which subsequently appear in thoracic duct lymph. Furthermore, it might be expected that if the 2 to 5 per cent labelled large and medium lymphocytes which are present during the first few hours should continue their expected developmental sequence, a much higher percentage of labelled small lymphocytes would result. In fact, when the labelled small lymphocytes are present in greatest numbers, the total percentage of labelled lymphocytes is about the same or slightly less than the percentage at the earlier intervals. The failure to obtain a larger percentage of labelled small lymphocytes may be related to the greatly diluted TTH resulting from continued division of the labelled precursors. For example, a large lymphocyte effecting a reduction of 100 silver grains would be interpreted as a heavily labelled cell. Assuming that such a cell was in the second generation according to the schema of Sainte-Marie and Leblond (1958), the derived small lymphocytes might be expected to produce an average of one and a half grains within the same period of exposure. It is likely that many cells of this category would be overlooked or not interpreted as labelled cells. Another possible complicating factor relates to the geometry of the cells in smear preparations. Large lymphocytes could conceivably flatten more than a small cell and thus would provide for more effective reduction by the weak beta particles of tritium in the larger cell. Thus, it seems possible that many small lymphocytes arising from lightly labelled precursors may not register radioautographically under the usual conditions of exposure.

The reliability of attempts to determine accurately the numbers of newly formed small lymphocytes may be questioned on the basis of the lymphocytosis experiments. In two guinea pigs, for example, the blood leucocyte count was increased two to threefold; presumably the production rate was increased as judged from proliferative activity in the lymphopoietic centres. It might

be expected that a large fraction of these newly formed cells would be labelled. Although there was an increase in the labelled cell population in all tissues studied, the increase was not of the magnitude expected. It is possible, however, that the TTH injections were not made at the right times to allow maximal labelling.

A limited number of transfusion experiments (lymph or blood) showed clearly that some small lymphocytes recirculate from blood to lymph, but the numbers of labelled cells administered was small and the periods of sampling too brief to estimate percentages. It would appear, however, that there was no predilection for labelled cells to appear in thoracic duct lymph or in lymphoid tissue. In fact, when considered with respect to the size of the tissue sampled, the greatest numbers of labelled [illegible] evidence, however, that the transplanted labelled cells seen in the bone marrow or other tissues were undergoing division or transformation

It is believed that the exchange blood transfusions provided the most physiological means to introduce labelled lymphocytes from one animal to another; little time was lost in transfer and *in vitro* manipulations were reduced to a minimum. Furthermore, these experiments provided for a more satisfactory identification of labelled cells in the recipient.

In evaluating the results of these transfusion experiments, it is perhaps important to consider the fact that small amounts of heparin were introduced with the blood or lymph and that the recipient rats were lightly anaesthetized with ether during the brief period of transfusion Ether and heparin have each been reported to alter capillary permeability.

Even though the present studies show that some labelled lymphocytes recirculate from blood to lymph, the observations are

not in accord with a continuous recirculation of these TTH-labelled cells between blood and lymph nor are they in accord with a long blood lifespan for these cells. Admittedly, the number of experiments is too small to be conclusive; nevertheless, the inability to find labelled cells in blood or lymph of one animal 48 hours after transfusion would suggest that their circulating lifespan is not more than a few days in length. This is in accord with earlier observations for the guinea pig (Everett, Reinhardt and Yoffey, 1960).

Summary

The advantages and the possible shortcomings of using radioisotopic methods for studying blood cell formation have been presented. The chief advantages concern the specificity and sensitivity of the methods. The primary disadvantage stems from the possibility of radiation damage sufficient to alter development or lifespan of cells.

Special consideration is given to the radioautographic technique for studies of haemopoiesis. This includes an account of improved technical applications for the radioautography and subsequent staining of tissue smears.

An account is given of certain aspects of lymphocytopoiesis in the guinea pig and rat as revealed by use of [^{3}H]thymidine.

After a single dose of [^{3}H]thymidine, labelled large and medium-sized lymphocytes are present in lymph, blood and the lymphopoietic tissues at the earliest intervals sampled. Labelled small lymphocytes first appear during the fourth hour and increase in numbers as the percentage of labelled large and medium lymphocytes decreases. The sequence in which the labelled cells appear, together with the results of *in vivo* culturing of lymphocytes using Millipore chambers, shows the developmental sequence of lymphocytes to be large→medium→small.

Labelled small and occasional medium-sized lymphocytes appear in thoracic duct lymph, bone marrow, spleen and lymph

... of lymph or blood (exchange transfusion), ... which received [^{3}H]thymidine.

... lymphocytes, primarily small, have been ob... of guinea pigs and ...

The detection of lightly lab... midine administration is not interpreted to imply ... lymphocytes are long-lived: rather these lightly labelled cells reflect the dilution of the [^{3}H]thymidine label through the successive mitoses of precursor cells.

Acknowledgments

Acknowledgment is made to Lotta Matson, Chief Laboratory Technician, for her technical supervision and special contributions to the progress of the study, to Joyce Moon and Judy Lambert for their technical aid, and to Mrs. M. Nomura and Mrs. L. Krismer for secretarial assistance.

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DISCUSSION

Leblond: It is not known why so few small lymphocytes are labelled in the thymus. One hour after [^{3}H]thymidine injection, Mr. E. Kallenbach in my department found that 59 per cent reticular cells, 64 per cent large lymphocytes, 30 per cent medium lymphocytes, but only 0·28 per cent small lymphocytes, are labelled. This finding would be consistent with the widely held belief that small lymphocytes do not divide. However, 1 to 2 per cent small lymphocytes are found at various stages of mitosis in the thymus; and, while a few of these mitoses end in pyknoses, most of them lead to recognizable anaphases and telophases.

It was then thought that the paucity of labelled small lymphocytes was due to a very rapid DNA synthesis prior to the mitoses of these cells. If this were the case, the number of silver grains over those cells which are labelled would be high. However, the grains were found to be less numerous over labelled small lymphocytes than over other cells in the thymus.

Another possible explanation was a failure of DNA synthesis in small lymphocytes, as they might undergo mitosis with little or no previous DNA synthesis In such a case many small lymphocytes would contain less than the diploid amount of DNA. However, measurements of DNA in lymphocytes by Hewson Swift (1950. *Physiol. Zool.*, 23, 169) revealed that thymic lymphocytes have a diploid DNA content, with a few tetraploid cases. Hence, this explanation is not satisfactory either

Yoffey One might also mention Petrakis (Petrakis, N. L. [1953]. *Blood*, 8, 905), who says that from spectrophotometric analysis the small lymphocyte has the full number of chromosomes.

Cronkite: The cogent thing is the matter of the weak labelling of the small lymphocyte, with which I concur. I also concur with Prof. Leblond's observations of the percentage labelling of the large, medium and small lymphocytes However, we do not see mitoses in the cells which we call small lymphocytes. There may be a technical reason—we use smears and he uses sections Dr. Trowell, in his observations on cell suspensions from lumbosacral lymph nodes of the small rat, was also unable to find mitosis in the small lymphocytes.

I do not believe there is any good evidence for a metabolic abnormality or difference of the small lymphocytes as far as incorporation of thymidine goes. They do become labelled, and the question is: why do they not become labelled more intensively? One could say that this is a non-dividing cell, hence on an average its label must be half that of whatever the precursor may have been, and certainly I do not know what this is. Next, it is a dense cell. Our evidence indicates that thymidine enters by diffusion, so that the amount that can get in during the time that the thymidine is available is a function of the surface area of the cell. So this cell, if it ever does synthesize DNA, gets less labelling material during the time that the label is available.

The range of the β particles from the tritium is so short that if equal amounts of label are present in two cells and one cell is spread out to the thickness of 0·5 μ and the other to 2 μ, there will be apparently eight times the amount of label, whereas in reality the label is identical The differences are explicable strictly on the geometry of the situation. [illegible] small lympho- [illegible] ld [illegible] en one would label successive groups of cells that are coming into DNA synthesis. We have done precisely this and one can actually get a rather high percentage of small lymphocytes labelled. We have got them up to 40 per cent, but it takes 22 days with injections every 12 hours to do it. In other words, everything indicates to me that this is a non-dividing cell which does not synthesize DNA. I have no idea what its precursors are. It must live a really long period of time in the rat, perhaps an average lifespan of sixty to seventy days, and in man, using the same deduction and computations, we come out with greater than a hundred days for the lifespan of the small lymphocyte. Hamilton and Ottesen came to the same conclusion much earlier from other types of studies (Hamilton, L. D. [1956] *Nature (Lond.)*, 178, 597; Ottesen, J. [1954] *Acta physiol scand.*, 32, 75)

Leblond: Perhaps, before continuing this discussion, it should be emphasized that small lymphocytes have to be precisely characterized. In the paper I shall present later, written in collaboration with Dr. Sainte-Marie, they are defined as cells with a nuclear diameter measuring less than 4·5 μ in sections. We feel cells defined in this manner

undergo mitotic division. (The mitotic figures are dense and may have been missed by many authors.) The evidence indicates that the cells arising from these mitoses are still small lymphocytes, but no longer have the ability to divide.

To return to the problem of why so few small lymphocytes are labelled soon (1 hour) after [^{3}H]thymidine injection—a fact which is true whether smears or sections are used—the difference between small lymphocytes and other cells may be shown in a different manner. My collaborator Dr. B. Messier found that the ratio of the percentage of labelled cells to the percentage of dividing cells usually varied between 4 and 12 (for instance, 5, 9, 4 and 12 respectively for oesophageal epithelium, colonic epithelium, parenchymal cells of liver, and acinar cells of pancreas). In the thymus, the ratio was 13 for reticular cells, 5 for large lymphocytes, 1·4 for medium lymphocytes, and only 0·1 for small lymphocytes (E. Kallenbach).

O. F. Nygaard and R. L. Potter (1959. *Radiat. Res.*, 10, 462), using chemical methods, observed a poor incorporation of [^{14}C]thymidine in thymus as compared to spleen and small intestine. Their evidence led them to conclude that the pool size for one or more of the intermediates between the injected precursor and DNA was greater in thymus than in other tissues. However, this interpretation would require that all thymus cells be poorly labelled. The figures just quoted indicate that this is true only of small and to a lesser extent medium lymphocytes. Nevertheless, there is a possibility that a large pool of thymidine-DNA intermediates occurs, but only within the cell body of small and to a lesser extent medium lymphocytes. Such a hypothesis would account for the low labelling of these cells.

THE USE OF TRITIATED THYMIDINE IN THE STUDY OF HAEMOPOIETIC CELL PROLIFERATION*

EUGENE P. CRONKITE, VICTOR P. BOND, THEODOR M. FLIEDNER,† and SVEN-ÅGE KILLMANN

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FOLLOWING the development of tritiated thymidine independently in the United States (Hughes, 1957) and in Belgium (Verly, Firket and Hunebelle, 1958), this compound has been used extensively in the study of cell proliferation and deoxyribonucleic acid (DNA) replication in plants and animals. It has been applied in: chromosomal replication (Taylor, Woods and Hughes, 1957); radioautography of polytene chromosomes (Ficq and Pavan, 1957); studies of nucleic acid and protein synthesis (Ficq, 1958); general labelling of mammalian tissues (Hughes *et al.*, 1958); problems in leucocyte transfusions (Brecher *et al.*, 1958); *in vitro* synthesis of DNA by some circulating cells of normal human blood (Bond *et al.*, 1958, 1959*a*); the study of kinetics of cell turnover in the small intestine (Quastler and Sherman, 1959); cell renewal in gastrointestinal tract and other tissues (Leblond and Messier, 1958; Leblond, Messier and Kopriwa, 1959), general study of human and animal haemopoietic cell proliferation (Cronkite *et al.*, 1958, 1959*a*, 1960; Bond *et al.*, 1959*b*; Maloney and Patt, 1958; Patt and Maloney, 1959*a*, *b*); study of lymphocyte function, fate and lifespan (Yoffey, Everett and Reinhardt, 1958,

* Work supported by the U S Atomic Energy Commission.

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1959; Gowans, 1959*a*, *b*; Schooley, Bryant and Kelly, 1959; Everett, Reinhardt and Yoffey, 1960); study of nucleic acid metabolism (Lajtha and Oliver, 1959; Woods and Taylor, 1959; and Fitzgerald and Vinijchaikul, 1959); tissue culture (Verly, Firket and Hunebelle, 1958; Painter, Drew and Hughes, 1958; and Painter and Drew, 1959); in the study of the turnover and metabolism of injected thymidine (Rubini *et al.*, 1960); and in the study of turnover rate of human neoplasms (Johnson *et al.*, 1960; Cronkite *et al.*, 1960). No attempt has been made to cover all of the rapidly expanding literature in this area, and only those papers which bear directly or indirectly upon the problems of application and interpretation of radioautographic studies on haemopoietic cell proliferation have been cited. In an earlier publication (Cronkite *et al.*, 1959*a*) the assumptions and premises upon which analysis of [^{3}H]thymidine labelling data are based were discussed. Briefly, it is assumed that:

(1) The tritium and labelled thymine are largely non-exchangeable after DNA is tagged.

(2) DNA turnover is due solely to cell division and death.

(3) Reutilization of labelled materials is insignificant.

(4) DNA synthesis in general destines a cell to divide again.

(5) *In vitro* labelling determines the proliferative potentials of the cells.

(6) *In vivo* labelling makes possible the study of the kinetics of cell proliferation.

(7) There is no perturbation of the normal cell generative cycle by radiation injury from the [^{3}H]thymidine incorporated into DNA or from the formation of labelled degradation products.

In this paper the following are presented:

(1) A summary of the sequence of events in granulocytopoiesis in normal human marrow after labelling by [^{3}H]thymidine.

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days. Label was fixed and detectable in granulocytic precursors within one minute. The grain count was maximal by one hour. During the first three days the label spread into the non-dividing compartments. The labelling simultaneously decreased in intensity with successive cell divisions, thus facilitating an estimate of the time between successive mitoses for various cell types. Radioautographic studies have shown that the group of cells called haemohistioblasts, haemocytoblasts, large and small naked nuclei (reticulum cells?), large and small reticulum cells, and myeloblasts had labelling indices from 35–93 per cent. Of course the number of these cells seen was relatively small. Promyelocytes had a labelling index of 76 per cent and large myelocytes 68 per cent. The percentage of labelling of these cells remained relatively constant during the first three to five days after injection, whereas the grain count diminished in what appeared to be an exponential fashion. However, as the observed grain count approaches the background an apparent second component of the curve appears, partly because one counts whole grains and the apparently labelled population diminishes in size.* The half-time of decrease in the average grain count is in all probability primarily a function of the average time between successive mitoses. On this basis one can estimate generation times for the myeloblasts to be between 1·3 and 1·6 days (see Fig. 1A). The generation time for myeloblasts is approximately 1·3 days. There is so much scatter in the data for promyelocytes that it is not feasible to fit a curve, but the range in generation time is between 1 and 2·5 days. Large myelocytes start with a labelling percentage of about 70 and the grain counts diminish with a half-time of about 2·25 days

* Each electron emission which hits the emulsion produces on an average a little more than one grain. When average grain counts are based on the visibly labelled population, distortions are introduced as the mean count approaches zero. The more heavily cells are labelled initially, the longer it takes this group to reduce its intensity by successive mitoses to a level where, on the average, there will be no emission during the exposure time. This can be corrected in theory, but it is difficult in practice.

(2) Preliminary work on function of the "mononuclear" circulating blood cells of the rat during induction of sterile inflammation.

(3) Status of present knowledge on the production of radiation injury in lymphocytes by [^{3}H]thymidine.

The sequence of events in erythropoiesis and in the appearance of labelled granulocytes in the blood has been presented and analysed previously (Bond *et al.*, 1959*c*).

Materials and methods

(1) Granulocytopoiesis in man.

The patient, [^{3}H]thymidine, radioautographic techniques, and metabolism of [^{3}H]thymidine have been described (Rubini *et al.*, 1960; Bond *et al.*, 1959*a*; and Cronkite *et al.*, 1958).

(2) Labelling of lymphocytes by repetitive injections and rôle of lymphocytes in sterile inflammation.

[^{3}H]thymidine was administered in doses of 0·5 μc/g. (specific activity 1·9 c/m-mole) every 12 hours for three days. At various time intervals before and after induction of a Selye inflammatory pouch, animals were killed for radioautographic studies. Details will be published (Witschi and Cronkite, 1960; Cronkite *et al.*, 1960).

(3) Radiation injury of lymphocytes by [^{3}H]thymidine.

[^{3}H]thymidine was given in a single injection in graded doses up to 20 μc/g. (specific activity 1·9 c/m-mole) and animals were killed after five hours for study of the number of pyknotic small lymphocytes (Trowell, 1952). In addition injections of [^{3}H]thymidine at a dose level of 0·5 μc/g. were given every 12 hours, and animals were killed intermittently for radioautography studies of pyknosis.

Results and discussion of granulocytopoiesis in man

Bone marrow aspirations were commenced one minute after the injection and continued at successively longer intervals for 15

Fig 1 Decrease of mean grain count with time in various primitive progenitor cells (1A) and in large myelocytes (1B). Increase in percentage of labelled cells and maximal labelling index in small myelocytes (1C) and in metamyelocytes (1D).

(54 hours) (Fig. 1B). Small myelocytes start with a labelling index of about 20 per cent which climbs to 72 per cent at the rate of 3·2 per cent per hour (Fig. 1C). After the maximum percentage of labelling is attained the grain count diminishes, with a half-time of 2·4 days (60 hours) very similar to the decrease for the large myelocytes. Metamyelocytes are not labelled initially. Labelling first appears in the metamyelocytes three hours after injection and initially climbs at a rate of about 4 per cent per hour, attaining a labelling of about 65 per cent eventually (Fig. 1D). In Fig. 1C, D, is shown the increase in labelling of metamyelocytes and small myelocytes. In Fig. 2A, B, C, is shown the sequence in labelling of the metamyelocytes, band and segmented neutrophils in the bone marrow. It takes three hours for the metamyelocytes to label, 12 hours for the bands and 24–36 hours for the segmented neutrophils to label. Labelled band and segmented neutrophils appear in the blood after two to three and three to four days respectively. The sequence in the peripheral blood has been reported previously (Bond *et al.*, 1959*c*) and will not be commented upon here.

In the small lymphocytes observed in the marrow no labelling was seen initially, indicating that the small lymphocyte may be a non-dividing cell at least when it is resident in the bone marrow. After six hours labelled small lymphocytes began to appear. After three days the labelling index was up to about 8 per cent, with a constant grain count of about 2·5.

Much information on the time parameters of the cell generation cycle can be extracted by an analysis of the surge of cells from a labelled dividing compartment to a non-dividing compartment, as for example the appearance of labelled metamyelocytes. This general problem has been considered earlier (Cronkite *et al.*, 1958, 1959*a*, *b*; Bond *et al.*, 1959*c*), and will not be discussed in detail here. Cell proliferation can be compartmentalized and models constructed for apparent "steady state" equilibria with their largely unknown regulatory mechanisms that control

Fig. 1 Decrease of mean grain count with time in various primitive progenitor cells (1A) and in large myelocytes (1B). Increase in percentage of labelled cells and maximal labelling index in small myelocytes (1C) and in metamyelocytes (1D).

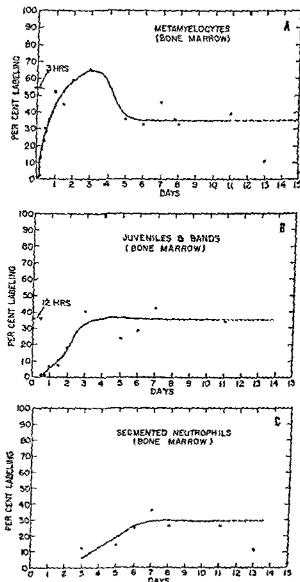


Fig 2. Appearance of labelled cells in the non-dividing compartments of metamyelocytes (2A), juveniles + bands (2B) and marrow segmented neutrophils (2C).

population size by increasing or decreasing flux across compartment borders upon physiological demand. It is recognized that these systems are not directly comparable to chemical steady state systems. However certain general principles can and have been applied (Bond *et al.*, 1959*c*). Next, one can analyse by graphic inspection the per cent labelling curves and extract fairly accurate information on the duration of various portions of the cell generation cycle. For example, it takes three hours for the first labelled metamyelocytes to appear. This sets a minimum time of three hours for premitotic rest (R_2) and mitosis of myelocytes. As cells enter, the per cent labelling increases and will continue to increase for a period equal in duration to time for DNA synthesis (t_s) of the immediate precursor. The per cent labelling attained will be determined in part by the time to be spent in the compartment entered. If this time is greater than the average t_s 100 per cent labelling will not be attained since cells not in the DNA synthesis period at the time of labelling will always be present, as well as labelled cells, in the non-dividing compartment. A graphic analysis of these time parameters is under study and as yet is incomplete (Cronkite *et al*, unpublished).

It appears that approximately 22 hours is the upper limit for renewal of metamyelocytes. Since the non-dividing metamyelocytes, juvenile bands and segmented neutrophils appear in the ratio of 1·0:1·4:1·6, one can estimate the average time in each stage as 22, 30·8 and 35 hours respectively for a total maturation time of about 87 hours Since the relative sizes of these compartments are proportional to time in the compartments one can compute from the average time of six to eight hours spent in the peripheral blood (Mauer *et al.*, 1960) that the immediately available reserve of mature neutrophils is of the order of four to six times the number circulating, and the total reserve of non-dividing maturing granulocytes that can be mobilized upon severe demand is about ten to fourteen times the amount of freely circulating granulocytes. These estimates are very rough and

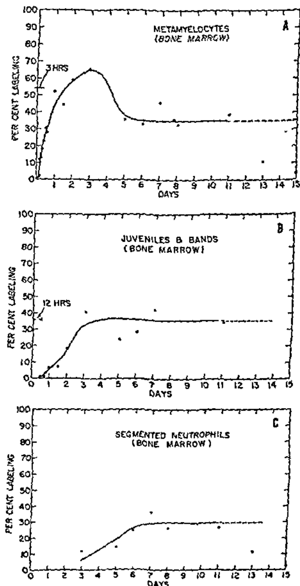


FIG. 2. Appearance of labelled cells in the non-dividing compartments of metamyelocytes (2A), juveniles + bands (2B) and marrow segmented neutrophils (2C).

duction line tends to resemble a "pipe-line" with surprisingly little straggling. The system appears to be open-ended with granulocytes disappearing into the tissues or being disposed of somewhere. It is highly suggestive that there may be a feed-back mechanism, related to disposal of the mature cells, by which a

Table I

COMPARISON OF BONE MARROW TIME PARAMETERS

	[³H]*thymidine*		*Mitotic index*
Primitive progenitor cells	1·32–1·57*		
Myeloblasts	1·32*		0·83–1·64†
Promyelocytes	1·0–2·5*		1·40–1·86†
Large myelocytes	2·25*		2·43–2·67† (Large and small myelocytes)
Small myelocytes	2·40*		
Metamyelocytes	0·92†	3·71† (Metamyelocytes to marrow segmented neutrophils)	5·38–5·92† (Metamyelocytes to marrow segmented neutrophils)
Juvenile bands*	1·28†		
Marrow segmented neutrophils	1·51†		

* [illegible] estimated from the half time of decrease in mean [illegible]

[illegible]

most important in the last proliferating compartment, i.e. the myelocyte compartment. [illegible]

[illegible]

calculated as 1·3 days

signal is transmitted from the dying cells to the production line to increase the production rate upon demand. However, diverse studies to date along these lines have come to little except for the demonstration of mechanisms for the mobilization of the rather sizeable intramedullary pool (discussed earlier) of metamyelocytes to segmented neutrophils. The diminution in the size of this granulocyte compartment inside the bone marrow cavity of fixed volume will produce a dilatation of the sinusoids by circulating

dependent upon the accuracy of the determination of the average duration in the blood and of the relative sizes of bone marrow compartments.

In considering the preceding data it must be recognized that the serial studies have been performed on only one human being, apparently in normal haemopoietic equilibrium. Spot checks on one other patient in haemopoietic equilibrium revealed essentially the same "flash" labelling index but serial studies were not performed so no comment on the time trend in the marrow can be made. However the sequence in the peripheral blood was essentially the same. Of considerable interest are the comparisons of the computed time parameters from mitotic index (Killmann *et al.*, 1960) and labelling by di-isopropylfluorophosphate ($DF^{32}P$) of the granulocytic series (Mauer *et al.*, 1959, 1960). In Table I is shown a comparison of the time parameters (Killmann *et al.*, 1960). The closeness of the computations from different sets and types of data is striking. In respect to the production and life cycle of the granulocyte in human beings certain fairly clear conclusions can be drawn. The *upper limit* of the *average stay* in the peripheral blood has been shown to be of the order of two days (Bond *et al.*, 1959*c*). The data of the Utah group using $DF^{32}P$ labelling (Mauer *et al.*, 1959, 1960) clearly indicate that the disappearance from the circulating blood is probably random, with a half-time of about six to eight hours, and that the labelled circulating cells very promptly come into equilibrium with a "marginal pool" of leucocytes on the vascular endothelium The size of the "marginal pool" is about equal to the number of the circulating granulocytes. The marginal pool can be released into the freely circulating blood by the appropriate stimulus, such as intravenous injection of adrenaline. The existence and size of an extravascular pool of granulocytes is still debatable. However, from our data (Bond *et al*, 1959*c*, Cronkite *et al.*, 1959*a*, *b*; and Mauer *et al.*, 1959, 1960) there is no evidence for any significant return of leucocytes from an extravascular pool. The entire pro-

donors, and by studying transformation and migration of cells, labelled in one part of the body while circulation was occluded, to the occluded area after the circulation is re-established. To date these studies have produced evidence for migration of monocytes and all types of lymphocytes to marrow, other lymph nodes, spleen and thymus. Appearance of labelled cell types suggests *conversions to plasma cells, reticulum cells and possibly to pro*myelocytes in the marrow. There is no evidence as yet for large-scale conversion to granulocytic precursors and no evidence for conversion to red cell precursors. If this occurs normally it must represent but a small fraction of new cell production. To date the labelling studies have not supported the concepts of Yoffey and others (reviewed, Yoffey and Courtice, 1956) that the small or any lymphocyte significantly contributes to red cell or granulocyte production. However, we recognize that negative *results do not constitute proof that such may not be the case to* some degree. In contrast to the difficulty of establishing quantitative evidence for migration to and cell transformation in the blood-forming organs in normal haematopoiesis it has been easy to demonstrate the migration of all types of circulating leucocytes into areas of sterile inflammation with probable significant transformation of large mononuclears into tissue macrophages and ultimately to fibroblasts (Witschi and Cronkite, 1960).

The Selye pouch, sterile inflammation technique was used in *three manners. First, pouches were initiated and at various time* intervals thereafter a single injection of [^{3}H]thymidine was given for "flash" labelling and the animals killed for study one hour later. Second, haemopoietic tissues of rats were labelled by injections of [^{3}H]thymidine every 12 hours for three days, with each injection containing 1 μc/g. (specific activity 1·9 c/m-mole), to produce maximal labelling of mononuclear cells; pouches were made two hours after the last [^{3}H]thymidine injection and animals killed at 12, 24, 48, 72 and 96 hours. Third, pouches were started and [^{3}H]thymidine given in the same dosage every

blood. It is not inconceivable that there are local stretch-tension signals within the sinusoidal walls that fire the signal for greater production. The mechanism of regulation of production must remain a subject for experimental study since so few facts are known today. However, there may be some common denominator between red cell, granulocyte and platelet production for there is a disease of the steady state equilibrium in which all three are being overproduced simultaneously—namely polycythaemia vera. In this situation it appears that the red cell production is progressively increasing in rate (red cell mass steadily or intermittently expanding) and leucocyte and thrombocyte production are set at a higher "steady state" level. Systematic study of the time parameters of this disease by the combined use of [^{3}H]thymidine and DF^{32}P labelling may throw considerable light on the problem of regulatory mechanisms.

Further refinements in time values and estimations of the duration of post-mitotic rest (R_1), DNA synthesis, and R_2 must await further studies and analyses of existing data. The studies performed to date have yielded useful information and indicate how to design new experiments.

The valuable data of Patt and Maloney (1959*a*, *b*) using [^{3}H]thymidine in dogs are consistent with our data on human beings. The provocative studies of Osgood in earlier years (see Osgood, 1959) and Ottesen (1954) and Kline (1959) using different techniques are not discussed since the intent has been to concentrate on the use of [^{3}H]thymidine. The comparative values of the diverse techniques have been well discussed (Stohlman, 1959).

Lymphocyte lifespan and function

Bond and colleagues (1959*b*) have presented preliminary data on conversion potentialities of labelled lymphocytes in parabiotic rats. These studies are continuing with cross-transfusion and direct transfusion of labelled cells from the thoracic duct of

laries. Approximately 90 per cent of haematopoietic cells were intensely labelled.

When the pouch was permitted to develop for 12 hours after cessation of the three-day labelling period, there was intense labelling of the cellular infiltrate of the pouch. About 99 per cent of the segmented neutrophils were labelled. Labelled mononuclears of all sizes were present and heavily labelled but much fewer in absolute number than the neutrophils. Labelling in the adjoining connective tissue was evident but the percentage was low. After 24 hours the labelling proportion was roughly the same but with a heavier exudate. The average grain count was 15. After 48 hours there was considerably more organization and the proportion of labelled granulocytes to labelled mononuclear cells had decreased. The grain count had decreased to about 10 per cell on the average. After 72 hours the proportion of labelled mononuclear cells was higher and there was a diversity in type of labelled mononuclears from all types of lymphocytes to labelled macrophages with amoeboid cytoplasm. Labelled mitoses were not seen. After 96 hours the grain count of labelled cells had decreased to less than 10 per cell The connective tissue cells (fibroblasts) that were being formed were in part labelled. Since only few fibroblasts in the pouch were flash labelled by 24 and 48 hours after the pouch induction, it is reasonable to postulate that labelled cells migrated into the area from the blood and were transformed into fibroblasts through a sequence of steps not yet established in these studies. Rather heavy labelling was still present in all of the haematopoietic tissues and leucocyte concentrates of the blood.

Cumulative labelling of pouches after initiation of the pouch

After 12 hours a small number of labelled mononuclears similar to labelled cells seen in the peripheral blood were found in the inflammatory exudate. After 24 hours the cell density and label-

12 hours for 12, 24, 48, and 72 hours to cumulate labelling in the area, and the animals were killed within six hours after the last injection.

Flash labelling after inflammatory stimulus

With "flash" labelling injection and pouch simultaneously and killing in one hour no labelling was seen in the pouch area. Hence, within the availability time of [^{3}H]thymidine, DNA synthesis had not been instituted. At 12 hours the flash labelling demonstrated that less than 1 per cent of the mononuclears in the area were labelled. However, some had 20–40 grains overlying the nucleus, indicating that either DNA synthesis had been initiated in the area in pre-existing cells or that cells that were labelled elsewhere had migrated into the area. A very rare fibroblast was intensely labelled. Flash labelling 24 and 48 hours after pouch induction showed a very low labelling of the fibroblastic tissue. Mitotic figures were seen but none were labelled nor was labelling expected (one hour is less than the premitotic rest period). At 48 hours labelled endothelial cells were seen in a small percentage.

Sequence after maximal labelling of haemopoietic tissues before initiation of pouch

Mononuclear cells of the peripheral blood were intensely labelled. Over 50 per cent of the large and medium lymphocytes were labelled after the 72 hours of labelling. In some instances labelling was close to 100 per cent. Approximately 90 per cent of the segmented granulocytes were labelled. In the connective tissue, away from the area where pouches were formed, there was very little labelling although an occasional fibroblast and perimysial connective tissue cell with 10–20 grains per cell was observed. In the area where pouches were formed a rare endothelial cell was labelled and a variety of "fixed tissue" cells were labelled in a low percentage, particularly in association with small capil-



Fig. 3A A labelled mitotic figure in connective tissue 72 hours after cumulative labelling

3B—A labelled mast cell and diverse labelling in the wall of inflammatory pouch

3C—Labelled endothelial and perivascular cells

ling had increased. Both granulocytes and mononuclears were labelled. After 48 hours (five injections) many labelled endothelial cells were seen. About 80–85 per cent of the inflammatory cells were labelled. In the blood vessels adjoining the inflammatory pouch there appeared to be a heavy concentration (greater than in blood at large) of labelled mononuclears within the lumen, but none were observed in the act of traversing the vessel walls. After 72 hours (six injections) up to 90 per cent of the cells in the inflammatory wall were labelled with average grain counts of about 20. Examples of labelled cells are seen in Fig. 3A–C.

These studies are not conclusive. However, if all of the cell proliferation in an inflammatory area was derived from cells that existed there prior to institution of the inflammatory process one would expect a high percentage to be labelled after flash labelling of the pouch at different time intervals. When the haematopoietic tissues were maximally labelled before institution of the pouch one found increasing labelling of the mononuclears in the exudate and eventually some labelled fibroblasts. This is strong evidence for migration of cells from the blood with transformation. Since the intensity of the label decreased by about one-half after about 24 hours, one is inclined to believe that the migratory cells also divided. Not only do these migratory inflammatory cells divide but apparently they are transformed in part at least to macrophages and fibroblasts, thus confirming the indirect studies of Allgower (1956) on colchicine build-up of mitotic figures in wounds. MacDonald (1959), using [^{3}H]thymidine labelling in wounds of rats, concluded that fibroblasts arise only from fixed connective tissue cells adjacent to the adventitia of vessels and then migrate to area of need. He interprets his data as indicating that fibroblasts do not arise from blood cells However, MacDonald does not give enough data on his experimental design, e.g. time from injection of [^{3}H]thymidine to biopsy, to interpret his observations. It appears that our data support, but do not prove, Allgower's belief of migration of mononuclears with mitosis and

transformation into fibroblasts. Neither our experiments nor MacDonald's are conclusive evidence for the origin of fibroblasts. We agree with MacDonald that there is proliferation both of the connective tissue cells around the adventitia and of endothelial cells, but perhaps the former arise also from blood cells. It may well be that fibroblasts have an apparent dual source, each of which arises from a more primitive mesenchymal cell. The best means by which our studies could be confirmed is by the transfusion of labelled thoracic duct lymphocytes from [^{3}H]thymidine labelled donors into rats with inflammatory pouches of varying ages followed by serial sacrifice. If labelled fibroblasts are then found one source is proved

Radiation injury of lymphocytes by injection of [^{3}H]thymidine

The problem of radiation injury by [^{3}H]thymidine was discussed in principle at the Salt Lake City Conference on cell proliferation (Stohlman, 1959). Radiation injury of cells in tissue culture by [^{3}H]thymidine was shown to be much greater than by equal amounts of tritiated water (Painter, Drew and Hughes, 1958). Johnson and Cronkite (1959) have shown that 20 μc/g. simulates the effect of 40 r. and 5 μc/g simulates the effect of about 10 r. in studying the radiation effects on spermatogonia; with less than 1 μc/g no effect was detected. Lymphocytes have a comparable degree of radio-sensitivity. The Trowell technique is a satisfactory quantitative measurement of radiation effects on the small lymphocyte. Many of the studies with [^{3}H]thymidine labelling of lymphocytes are concerned with proliferative potentials and transformation of lymphocytes into other cell types. Since radiation may drastically influence both proliferation and transformation capabilities in some subtle manner it appeared mandatory to investigate radiation injury of all types of lymphocytes following single and repetitive injections.

In Fig. 4 is shown the initial experiment on the effect of single graded doses of [^{3}H]thymidine *on pyknosis* of small lymphocytes compared to the effect of X-ray. By extrapolation 25 μc/g. simulates the effect of 75 r. whole-body X-ray. The interval between 0 and 5 μc/g. has not been investigated but there is no significant difference between the controls and 5 μc/g. Unfortunately the thick film technique of Trowell, though excellent

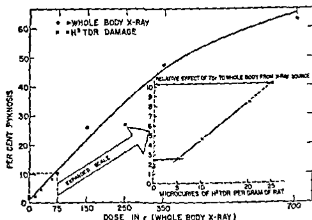


FIG. 4. Percentage of pyknotic small lymphocytes after single doses of tritiated thymidine compared to whole body X-irradiation. (H³TDR:—tritiated thymidine)

for its basic purpose, is very poor for radioautography and drastically reduces the efficiency of tritium because of the short path length of the ^{3}H beta particle. Labelled pyknotic nuclei of both large and small lymphocytes are found easily after a dose of 20 μc/g. but because of the thick film it is impossible to quantify. One also sees phagocytic cells laden with nuclear debris from time to time; again quantification has not been attempted. Labelling of the nuclear debris was not present but the cells were so deep in the film that labelling could have been missed. The maximal dose of radiation from the injection of [^{3}H]thymidine uniformly distributed in the body and converted to soluble uniformly dis-

tributed products would be about 0·012 rad/hour/μc ^{3}H or a total dose of 0·06 rad per mc injected for the five-hour period between injection and killing. One must assume that the concentration of the thymidine in DNA is responsible for the bulk of the observed radiation injury.

In Table II is shown the relative lack of effect from the repetitive injections of [^{3}H]thymidine (0·5μc/g. every 12 hours). However,

Table II

CUMULATIVE EFFECT OF DOSES OF 0·5 μc/g. OF [^{3}H]THYMIDINE EVERY 12 HOURS. RATS KILLED 1 HOUR AFTER LAST INJECTION

Time after first injection	*Cumulative [^{3}H]thymidine in μc/g.*	*Per cent pyknosis*
1 hour	0·5	1·75
12 hours	1 0	2·46
3·5 days	3 0	2·96
5·5 days	6·0	4 58
6·5 days	7·0	2·66
8·5 days	9 0	1·52
11·5 days	12·0	4·14
13·5 days	14 0	3·10
14·5 days	15 0	1·07
17·5 days	18·0	1·10

as Trowell (1952) has pointed out, pyknotic cells are removed relatively rapidly after radiation. They are visible promptly, attain maximum level at about five hours after whole-body irradiation, and then decrease in number. In the repetitive injection study it is entirely conceivable that the rate of removal of pyknotic cells is sufficiently rapid so that we could not detect radiation injury by this means. A search is under way for mitotic aberrations. It appears by the usual criteria of radiation injury that [^{3}H]thymidine in the doses used for single injection labelling and radioautography is relatively innocuous. However further searches should be made for more sensitive and subtle evidences of impairment of cell function. At the present time it appears that repetitive injections at a level of 0·5 μc/g. every 12 hours

produce little damage. However 12 hours is probably longer than the DNA synthesis period so that the same cells will not be relabelled until they have completed a division (halved their label on the average) and entered DNA synthesis again. After the second labelling the ^3H content will be 1·5 times the initial labelling on the average, and it is obvious that the maximum labelling will converge on 2·0, irrespective of the number of injections, so long as the time between injections is greater than the sum of time for DNA synthesis and availability time of the [^3H]thymidine. However the radiation dose from tritiated water will progressively climb as more will be produced than is being turned over since water has a biological half-time of about ten days. About half of the [^3H]thymidine is converted to tritiated water. Hence the concentration of tritiated water will progressively climb and may produce significant radiation effects by itself after repetitive injections.

Summary

In vivo labelling with tritiated thymidine followed by radioautography was used to study normal granulocytopoiesis in man, the destiny of circulating DNA-synthesizing mononuclear cells in rats and the origin of inflammatory cells in sterile inflammation in rats. In addition some preliminary data on the radiotoxicity of tritiated thymidine are given.

A kinetic analysis of human granulocytopoiesis based on the decrease in mean grain counts of cells capable of division and the percentile increment with time in the number of labelled cells in the marrow compartments consisting of cells incapable of division is presented. The half-time of decrease in the mean

cytoblasts, reticulum cells) is estimated to be between 1·32 and

1·57 days, and the generation time for myeloblasts, promyelocytes and large myelocytes at 1·32, 1–2·5 and 2·25 days respectively; these figures are preliminary and subject to later revision, particularly since they were not corrected for the perturbation by influx of labelled cells from preceding labelled compartments. The average turnover time of the compartment consisting of cells incapable of division (metamyelocyte to marrow segmented neutrophil) was about 87 hours; with the type of analysis employed this is the upper limit for the average time spent in this compartment.

Preliminary data on conversion potentialities of labelled lymphocytes in parabiotic and cross-transfused rats are presented; no evidence has been found for a conversion of lymphocytes to red cell precursors or for any large-scale transformation to granulocytic precursors.

Single or repeated injections of tritiated thymidine were given to rats before or after induction of a sterile inflammation. With single tracer injections at various times after the induction of inflammation very few cells were labelled. On the other hand, if the haemopoietic tissues had been maximally labelled before inflammation was induced, heavy labelling of all types of mononuclear cells and to a lesser degree of fibroblasts was present. These findings suggest that cells from the blood migrate to the site of inflammation; since the grain counts of the inflammatory cells decrease with time it appears possible that in addition cell proliferation takes place *in situ*. The data provide evidence but do not prove that fibroblasts arise, at least in part, from circulating blood cells.

Intranuclear irradiation effects of tritiated thymidine could seriously perturb the capacities of cells for proliferation and transformation The Trowell technique was used to estimate the radiation damage to small lymphocytes by tritiated thymidine as compared to the effect of whole body X-irradiation. Preliminary studies show no significant effect of single doses of 5 µc/g. rat, with single doses of 10 and 20 µc/g the percentage of

pyknotic small lymphocytes increases. By extrapolation 25 μc/g. simulates the effect of 75 r. whole-body X-irradiation. It thus appears that tritiated thymidine is relatively innocuous in the single doses (0·1–1·0 μc/g.) usually employed in studies of cellular kinetics. The effect of repetitive doses of tritiated thymidine is more difficult to evaluate; with injections of 0·5 μc/g. every 12 hours the number of pyknotic small lymphocytes rose until a cumulative dose of 6 μc/g. was attained. Following this a plateau was reached, perhaps even with a tendency towards a decline in the pyknotic count at the highest cumulative doses. A possible explanation is that an equilibrium is reached between the formation and removal of pyknotic cells in the intact animal.

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pyknotic small lymphocytes increases. By extrapolation 25 μc/g. simulates the effect of 75 r. whole-body X-irradiation. It thus appears that tritiated thymidine is relatively innocuous in the single doses (0·1–1·0 μc/g.) usually employed in studies of cellular kinetics. The effect of repetitive doses of tritiated thymidine is more difficult to evaluate; with injections of 0·5 μc/g. every 12 hours the number of pyknotic small lymphocytes rose until a cumulative dose of 6 μc/g. was attained. Following this a plateau was reached, perhaps even with a tendency towards a decline in the pyknotic count at the highest cumulative doses. A possible explanation is that an equilibrium is reached between the formation and removal of pyknotic cells in the intact animal.

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either in survival or in recovery of white count. Initially the transplantations were made one day after thymidine was given to the donor mice, and in a second experiment eleven days after thymidine.

Craddock: I am troubled by the possibility that there may be intracellular thymidine pools in certain types of cells. You stated that this possibility was either very small or even non-existent—is there good evidence that this is so in all types of cells you have observed?

Cronkite: If one follows the evolution of tritiated water, which is a metabolic product, it goes up to a maximum by 60 minutes and no more is formed. I am reasonably confident that labelled thymidine is not being diluted in a pool of any significant size, nor is it being incorporated into a form where it is no longer degraded to tritiated water if not incorporated into DNA. A very simple arithmetical computation of the number of disintegrations per minute that have been distributed between roughly 10^{14} cells in an average man after 9 mc injection shows that there must be some type of concentrating mechanism while material is available or one could not produce a radioautograph. [^{3}H]thymidine diffuses through the capillary bed and comes into equilibrium with total body water extremely rapidly. If the cell which it enters is synthesizing DNA it is rapidly incorporated into DNA, so that there apparently is a one-way street—it goes in and is trapped by the incorporating mechanism, thus there is a maximal diffusion gradient during the time label is available. If there were any significant pool of endogenous thymidine as such we could not get a radioautograph. Sample computations show this (Cronkite *et al.*, 1959*a*, *loc. cit.*).

Lajtha: There seems to be some difference between the overall specific activities depending on the label used and the organs investigated. With ^{32}P you get very high DNA-specific activities in the thymus—much higher than in liver, and higher even than in the intestine. With [^{14}C]thymidine or tritiated thymidine the thymus gives a very low specific activity, hardly above that for the liver. This in itself does not necessarily mean that the thymus contains a great thymidine pool; it may also mean that thymus cells have a very efficient pathway synthesizing their own thymidine and do not very much like the thymidine you inject. But we have been impressed with the differences between grain counts in different organs very

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DISCUSSION

Yoffey: Is it not possible to get some cell in culture which everybody accepts as a growing cell and which is capable of normally undergoing some differentiation in culture, and to apply known levels of thymidine there, perhaps on the lines that Firket (1958. *Arch. Biol. (Liège)*, 79, 1) did in his *in vitro* studies? It is obvious that if you get enough thymidine in you will cause cell death and pyknosis, but the critical point that we are all worried about is whether the smaller doses will just interfere with the normal cycle of developmental and differentiation changes

Cronkite. This has been done by R B. Painter, R. M. Drew and W. L. Hughes (1958, *loc. cit.*) and at the levels they were using they could find no perturbation of the normal cell cycle in the HeLa cell tissue culture, which is quite sensitive to radiation. They did notice that if you went up to the higher doses and started increasing specific activity you got injury and the injury went up with increase in the specific activity of the thymidine

Stohlman: In conjunction with Drs. W. W. Smith and G. Brecher I have done a few pilot experiments which bear on the question of radiation damage from thymidine. The effect of thymidine on bone marrow transplantation in irradiated mice was determined. Using 10 μc/g. in isologous mice we have not been able to detect any change

would still be of the same shape. I have reasonable evidence that very few cell populations are truly homogeneous. There are chronic stragglers in every population which get labelled and for some reason just stay there. I think therefore that the figure of 1·3 days is probably the upper limit, and the likely figure may be 10 or 20 per cent less. [illegible] there were [illegible]

Lajtha: I mean that this limit you are getting is due to the stragglers, which in fact did not divide during that time and therefore increase your grain count.

Stohlman: If they don't divide then their intermitotic time is infinity and 1·3 days would not be the upper limit.

Lajtha: It entirely depends on what your grain count distribution curve looks like in time. If it remains the same type of distribution curve, fair enough, but if you see that it broadens out with time, then that is a pretty good indication that the cells did not behave uniformly, and some of them divided once, some twice, and some not at all.

Cronkite: The distribution of the histograms of labelled granulocytes in peripheral blood moves over as one would expect, until there is an artificial diminution in the fraction labelled simply because with time and successive mitoses cells are produced with so little label that there are no disintegrations during the exposure period. It does not look as if there is a population of stragglers, and certainly after the first couple of days there are really no heavily labelled cells left in the marrow, such as are seen initially. There are a lot of alternative explanations on how to interpret the findings, but I do not think that in this case there is much straggling. In animals we have observed a small fraction of heavily labelled cells remaining in lymph nodes for as long as a week.

Loutit: Does this figure of 95 per cent labelling indicate that some cells are for 95 per cent of the time in the DNA synthetic period, or is there some other storage or artifactual phenomenon creeping in?

Cronkite: If they spend their lifetime in the marrow then 95 per cent of the time is spent, on the average, in DNA synthesis. If they come from somewhere outside and only synthesize DNA in the marrow, then this is not representative of the total life cycle of that particular cell.

Lamerton: Was this with a single injection?

Cronkite: Yes.

shortly, or three or four hours after a single injection of thymidine. The thymus and spleen do differ quite a bit.

Secondly, there are some cells which, at least for a while, may contain thymidine in their cytoplasm or nucleus in a form which is not DNA. I am referring to some hitherto unpublished observations of Prof. J. A. V. Butler, at the Chester Beatty Institute. He has incubated cells from mouse ascites tumours for one to five minutes with thymidine, washed them in Hanks' solution, and then determined the total radioactivity in the cells as well as the total radioactivity of the DNA content. Within the first five minutes up to 90 per cent of the total activity in these cells is *not* in the DNA. We were very interested in that because when we started irradiating these ascites cells and investigating the effects of irradiation on DNA synthesis, using thymidine with a rather high specific activity, we had a very nice 50 to 60 per cent depression of the DNA specific activity, or grain count over individual cells, following a dose of 2,000 r. Then we switched over to another source of thymidine from Amersham and did not get more than about 10 to 15 per cent depression. I am afraid we very unjustly accused Amersham of giving us a bad batch of thymidine. Then we started thinking about the specific activity problem, and we compared compounds with high and low specific activities. We found that the cells upon irradiation may release enough thymidine (or thymidine-like substance) to dilute our administered label by 50 per cent, which in thymidine was equivalent to about 1 to 2 per cent of the DNA content of the cells.

The other point I should like to raise is the grain count half-time you gave, Dr. Cronkite. Do you agree with me that this is an apparent generation time, and that in fact individual generation times could be quite a bit less than this if the cell population is not functionally homogeneous?

If you have, say, two populations of cells, one which divides very slowly and the other dividing every six or ten hours, the time will come when the overall grain count will go down to half of what it was at zero time, simply due to the fact that one (the fast population) went down 75 per cent, the other (the slow population) only 25 per cent. What one would like to see with a grain count half-time is not only the mean or median grain count, but a grain count distribution which

Cronkite: We have only tried to observe it, using these techniques.

Braunsteiner: But you think these are lymphocytes and not monocytes?

Cronkite: They are mononuclear.

Braunsteiner: But generally monocytes are found only in a small percentage. There are so many cells coming from the thoracic duct that one is rather inclined to say they are lymphocytes.

Cronkite: They are mononuclears of various sizes in the blood stream —they are not "small lymphocytes," I am certain of that.

Everett: Have you observed the persistence of heavily labelled lymphocytes for a long period of time in the peripheral blood?

Cronkite: No. In human beings the percentage of labelling of large and medium lymphocytes is at a maximum between the second and third days, and then the grain count decreases very rapidly.

Everett: Then how can you reconcile these observations with your statement that the small lymphocyte must have a long lifespan?

Cronkite: I think our data indicate that the small lymphocyte probably has a long lifespan. I do not think our data indicate that it can come from the medium lymphocyte. I think it comes from something else. As a clinical haematologist I am used to looking at classical smears [illegible]

[illegible] can tell lifespan from observing the blood.

Cronkite: If the cells of the blood are in equilibrium with everything that is in the body, one can deduce from what is observed in the blood some information about the total lifespan. I think it is quite logical to do that.

[illegible]

[illegible] of his ideas and data. If it is an open-ended system and they are just going out, again they should have a peak of high labelling, as the

Leblond: In the medium lymphocytes the percentage of labelled cells is about the same in thymus as in peripheral blood (30 per cent). If we assume there is a steady state, these cells would therefore appear to synthesize DNA during 30 per cent of their lifespan (or generation time).

Cronkite: A very interesting thing to me (although again anaesthesia may be a perturbing factor) is that in a thoracic duct-cannulated dog huge numbers of labelled cells are pouring out into a volume that we have a reasonable estimate of, namely the circulating blood. The equilibrium concentration is much below what it would be if they were circulating for any significant period of time. Whether they are just dying in the peripheral blood, or going out into the tissues, or going back into the lymph nodes, we cannot answer from this procedure. We do know that when we use the Selye pouch technique [illegible] six days, there is an out- [illegible] exudate. It looks, as far as we have gone using the [illegible] y pouch, as if a major source of the fibroblastic formation after three or four days comes from mononuclear cells that are circulating in the peripheral blood. There is something very similar in the peripheral blood to Prof. Yoffey's loading in the capillaries of the bone marrow. In the inflammatory area the capillaries are crowded with labelled cells and then they are out in the tissues and one sees labelled fibroblasts. There is no direct proof yet, but these observations strongly suggest that one produces the other.

Lajtha: I can confirm that *in vitro*. I have found exactly the same many times. There is a pure population of mononuclear cells *in vitro*, and quantitative transformation to fibroblasts.

Yoffey: What is the source of the mononuclears?

Lajtha. Peripheral blood, white cell concentrate (normal), infectious mononucleosis peripheral blood, or blast cell leukaemia peripheral blood.

Braunsteiner: Do you think that labelled lymphocytes leave the blood vessels and get transformed, as you have said, some to plasma cells and others to cells which are called histiocytes and which are found in granulation tissue?

Cronkite: They must leave the blood stream and go somewhere.

Braunsteiner: Can you observe this?

DIFFERENTIATION, PROLIFERATION AND MATURATION OF HAEMOPOIETIC CELLS STUDIED IN TISSUE CULTURE

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Maturation

ONE of the problems in our programme of experiments concerns the maturative activity of haemopoietic cells. To study this, the bone marrow cells are placed in a liquid medium. At the beginning of the experiment, and subsequently every 6 to 12 hours, the cells in the different maturation phases are counted (the absolute values per mm ^{3}). Since under such conditions haemopoiesis rapidly weakens while maturation continues, the first to decrease or disappear are the most immature cells, secondly the cells in the middle stage, and finally, those which are nearly mature.

Of the erythroblasts (Fig. 1), the first to disappear are the basophils, secondly the polychromatophils and finally the orthochromatics. An estimation of the length of maturation can thus be deduced from the above-mentioned behaviour. These experiments therefore permit one to compare the behaviour of the erythroblasts from normal bone marrow with those from marrows from various blood diseases, as well as to investigate the effect of various agents acting directly on the cells themselves *in vitro*

* Supported by the Blood Research Foundation, Washington, D C.

segmented neutrophil does—it goes up to 75 or 80 per cent when they surge out of the marrow into the peripheral blood.

Craddock: Isn't it possible that they could be held within the nodes for a long period of time before entering the blood?

Cronkite: Then we should see them labelled in the nodes, and we do not see that. In the secondary follicles you can see large lymphoblasts. A very high percentage of them are labelled; the reticulum cells and macrophages are labelled, but of the small lymphocytes only a very small percentage are labelled.

Leblond: If I may come back to Dr. Everett's question, you said that 3 or 5 per cent small lymphocytes may be labelled after a single injection of [^{3}H]thymidine. For how long did you keep that level in the blood?

Cronkite: In this case we only observed it for 15 days. I agree with both you and Dr. Everett on this. The grain count and percentage labelling are very low to begin with, and so far this has not been followed sufficiently long to see if the percentage labelling remains constant for weeks. This must be done.

Everett: We have also seen labelled small lymphocytes at long periods after thymidine but heavily labelled cells do not exist for more than a few days at most. We would interpret this to mean that these cells that persist and are weakly labelled are not of the same generation as ones we see earlier, and that they have arisen later from the labelled precursors. We might use the stem cell theory of Prof. Leblond for an explanation. For example, a large lymphocyte of the second generation, which might give a reduction of 100 grains, would produce definitive small lymphocytes that would have a potential of only one and a half grains each, and I think we might miss a lot of them.

Leblond: An important finding is that the small lymphocytes found within the epithelium of the intestine do migrate to the lumen. This was shown by M. A. Jassinowski (1925. *Frankfurt. Z. Path.*, 32, 238) who observed large numbers of lymphocytes in washings of exteriorized intestine in rabbits. This finding was confirmed by Mr. E. Kallenbach in the rat, since large numbers of mononuclear cells were found next to shed epithelial cells in various states of digestion in the intestinal mucus. This loss is large enough to play an important rôle in the metabolism of lymphocytes.

person. This seems to prove that the above-mentioned abnormal maturation is more closely bound up with the humoral surroundings in which the erythroblast is living, than with its intimate biology.

A quite different result has been obtained by experimenting with the erythroblasts from *hookworm anaemia* (Astaldi, Rondanelli and Troiano, 1951). In this disease, before treatment, it has been seen that there is a significant delay in maturation not only when the cells are kept in the serum from the patient, but also when they are kept in the serum from a normal subject. On the other hand, after treatment the maturation activity of erythroblasts appears to be practically normal. These results seem to suggest that the delayed erythroblastic maturation in hookworm anaemia is not entirely due to the severe lack of iron which occurs in this disease. It may also be due to biological alterations in the erythroblasts, connected with the presence of the parasite, even though this hypothesis is not the most prevalent at the moment.

Some other researches have been carried out on the erythroblasts from the three forms of *thalassaemia*: major, minor and minima. Such investigations (Astaldi, Tolentino and Sacchetti, 1951) showed a delay in the maturation process in the orthochromatic phase, with a slowing down in nuclear loss before the erythrocytic stage is reached. This delay is largely manifested in the most serious cases of the disease and especially in the major form Besides this, it appeared that the erythroblasts from thalassaemia show the same degree of maturation whether they are kept in the serum from the patient or in the serum from a normal subject In the same way, erythroblasts from a normal subject behave similarly in the serum from the same subject and in the thalassaemic serum.

From these results, it seems that the retarded enucleation in the erythroblast from thalassaemia major is due to something which has occurred in the intimate biology of the cell, rather than to the

The experiments on the *erythroblasts from normal bone marrow* showed that 18 to 24 hours are necessary from the basophilic to the polychromatic stage, 12 to 24 hours from the polychromatic to the orthochromatic phase, and 24 to 36 hours from the latter to the stage of reticulocytes. The entire cycle of normoblastic

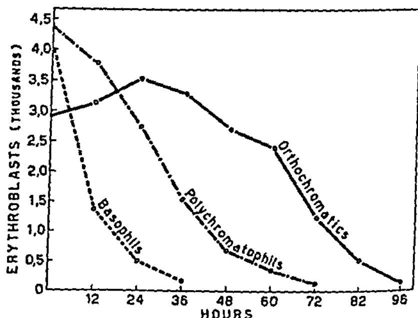


FIG. 1. The behaviour of the maturation phases of erythroblasts from human normal bone marrow during survival *in vitro*

maturation (to the adult erythrocyte) seems to need about one hundred hours

Researches carried out on the erythroblast from *primary hypochromic anaemia* (Astaldi, Mauri and Salera, 1950) showed a significant slowing down of the maturation process when these erythroblasts were kept in serum obtained from the patient But it may be observed that the same erythroblasts show a normal rate of maturation when they are kept in serum from a normal

concerning the problem of the normoblastic conversion of megaloblasts will be summarized.

Pernicious anaemia bone marrow in relapse, cultured in p.a. serum, remains megaloblastic. The same marrow, however, shows signs of normoblastic conversion when it is cultured in p.a. serum to which liver extract is added, as was first observed in this laboratory in 1946 (see Astaldi and Cardinali, 1957).

A normoblastic transformation has also been observed by cultivating bone marrow from patients with pernicious anaemia in serum from normal human subjects, or in serum from patients with pernicious anaemia after treatment (Astaldi and Cardinali, 1957; Lajtha, 1950; Callender and Lajtha, 1951; Franco and Arkun, 1951).

Marked signs of conversion are also observed when the p.a. bone marrow is cultured in p.a. serum to which folic or folinic acid is added (Lajtha, 1952; Callender and Lajtha, 1951; Astaldi, unpublished; Thompson, 1952; Franco and Arkun, 1951; Swan, Reisner and Silverman, 1955). However, the same marrow shows no significant conversion when it is cultured in p.a. serum to which thymine (Thompson, 1952) or vitamin B_{12} alone is added (Callender and Lajtha, 1951; Lajtha, 1950; Astaldi, unpublished; Thompson, 1952; Franco and Arkun, 1951). In order to obtain the normoblastic conversion, vitamin B_{12} has to be added, together with gastric juice (Callender and Lajtha, 1951; Lajtha, 1952). The same conversion seems to be obtained even when vitamin B_{12} is added to normal chick serum and chick embryo extract (see Astaldi and Cardinali, 1957), while both gastric juice alone (Callender and Lajtha, 1951; Lajtha, 1950) and chick embryo extract alone (Astaldi, unpublished), like vitamin B_{12} alone, are ineffective.

From the data reviewed, it can be seen that a conversion of megaloblasts to normoblasts *in vitro* has been observed by cultivating the megaloblastic marrow in the following media: (*a*) serum from normal human subjects, (*b*) pernicious anaemia serum

surrounding in which the cell is living. It should be noted, however, that such a phenomenon is not exclusively associated with thalassaemia, as it was observed also in other secondary erythroblastosis anaemias with hyperhaemolysis, such as leishmaniasis (Astaldi and Tolentino, 1949). This may suggest that we are dealing with a phenomenon which is not primary, but acquired. In any case, a retarded enucleation does occur in the erythroblast from haemolytic diseases of the newborn. In fact, when the erythroblasts present in the blood stream of this disease are kept *in vitro*, they reach the erythrocytic stage normally. This suggests that the later peripheral erythroblastosis may be compensatory.

Megaloblasts from pernicious anaemia in relapse show a delayed maturation as compared with normoblasts from normal bone marrow. In particular, when the pernicious anaemia (p.a.) marrow is cultured in p.a. serum, the whole cycle of megaloblastic maturation from the basophil to the orthochromatic stage requires not less than 72 hours, whereas the normoblast completes the same cycle in 48 hours.

This delayed maturation may be brought back to normal by the addition of agents to the p.a. serum used as a culture medium. It has been shown as a result that liver extract reaccelerates the slowed-down rate of megaloblastic maturation (see Astaldi and Cardinali, 1957) The same result (Fig. 3) has also been obtained with nicotinamide (see Astaldi and Cardinali, 1957). One should note, however, that while liver extract simultaneously restores normopoiesis, erythropoiesis remains megaloblastic with nicotinamide. The acceleration of megaloblastic maturation is therefore dissociable from the phenomenon of the normoblastic conversion of megaloblasts. It seems possible to conclude that the difference between megaloblasts and normoblasts consists not just in the slowing down of maturation, but also in some deeper biological characteristics, strictly connected with the morphological appearance of the cell.

At this point the results obtained by the *in vitro* culture method

on investigation of the local effect of inoculation of vitamin B_{12} directly into the pernicious anaemia bone marrow, it appeared that at the site of instillation the capacity for binding this vitamin is limited. Finally, by employing labelled vitamin B_{12} in a case of macrocytic anaemia, it was observed that there was a defect in vitamin B_{12} binding. Such a defect could be corrected by the administration of normal serum (Horrigan and Heinle, 1952).

By comparing both *in vitro* and *in vivo* observations it is possible to suggest that·

(*a*) A binding factor is needed in order that vitamin B_{12} may be utilized by megaloblasts both *in vitro* and *in vivo*.
(*b*) Such a factor is contained in normal serum, while it is lacking, or is insufficiently contained, in pernicious anaemia serum.
(*c*) The lack of a binding factor can be corrected *in vivo* by the administration of normal serum, and *in vitro* with normal serum as well as with embryo extract or gastric juice.
(*d*) The serum taken from a patient with pernicious anaemia treated with vitamin B_{12} does not show any lack of the factor referred to.

These observations in turn suggest the hypothesis (Astaldi and Cardinali, 1957) that in pernicious anaemia in relapse there is a lessening of a plasmatic factor which is needed for the utilization of vitamin B_{12} by blood cells (utilization factor). One may suggest that such a plasmatic factor may be the plasmatic binding factor (P.B F.), whose lessening may explain the lack of vitamin B_{12} action *in vitro*, where there is a very small quantity of serum. Such a defect can be obviated by adding protein substances contained in embryo extract, or in gastric juice. On the other hand, *in vivo* vitamin B_{12} may become biologically active, because there is not an absolute deficiency of P.B.F. owing to the great quantity of serum present in the entire body (in spite of the percentage lessening of P.B.F. in this serum) The local effect, too, obtained by the instillation of vitamin B_{12} into the bone marrow, may

taken from patients treated with vitamin B_{12}, (*c*) serum from patients with pernicious anaemia in relapse, + liver extract, (*d*) serum from patients with p.a. in relapse, + folic or folinic acid, (*e*) serum from patients with p.a. in relapse, + vitamin B_{12} + embryo extract, and (*f*) serum from patients with p.a. in relapse, + vitamin B_{12} + gastric juice.

No important effect on the conversion of megaloblasts to normoblasts was observed by cultivating megaloblastic marrow in pernicious anaemia serum alone, or with addition of one of the following substances: (*a*) vitamin B_{12}, (*b*) thymine, and (*c*) gastric juice.

The fact that no activity is exerted *in vitro* by vitamin B_{12} alone on megaloblasts growing in pernicious anaemia serum apparently contrasts with the therapeutical effectiveness of vitamin B_{12} when given to the patients by the parenteral route. Moreover, the lack of any effect *in vitro* from vitamin B_{12} seems especially to be in opposition to the effect of instillation of the drug directly into the bone marrow of patients with pernicious anaemia. In fact, there the vitamin induces the normoblastic conversion of marrow and corrects the cellular abnormality in ribonucleic acid (Horrigan and Vilter, 1950, 1951; Jarrol *et al*., 1951).

On the other hand, as mentioned above, vitamin B_{12} is effective *in vitro* when it is contained in normal serum, or added to pernicious anaemia serum in the presence of embryo extract or gastric juice. This proves that vitamin B_{12} is capable of acting *in vitro* only in the presence of an unknown factor which is contained in normal serum, in embryo extract, and in gastric juice, but which is lacking, or is contained insufficiently in pernicious anaemia serum. Since this factor cannot be vitamin B_{12} by itself, it is presumably a product capable of inducing a transformation or a binding of vitamin B_{12}. This is also supported by the fact that

The result (see Astaldi and Tolentino, 1949) was a delay in the maturation of normal erythroblasts following the addition to the cultures of a spleen extract from a case of lipoidosis. A delay in the maturation of erythroblasts was also observed in another case in which both the cells and spleen extract were obtained from the same patient with Cooley's anaemia.

Moreover, an increase in temperature above 37° and up to 42° accelerates maturation of the erythroblasts (Astaldi, 1951); up to 42°, there are very early signs of cellular damage, such as lysis. Colchicine in stathmokinetic doses (doses sufficient to stop all the mitoses at the premetaphase) does not interfere in the maturation from the basophil to the polychromatic stage, and it just slightly slows the subsequent maturation from the polychromatic to the orthochromatic phase. Moreover, it appeared (Mauri, 1951) that aminopterine inhibits maturation of both leucoblasts and erythroblasts, but to a higher degree in the latter; that urethane stimulates the polychromatic maturation of basophil erythroblasts; and finally, that methyl-bis(β-chloroethyl)amine does not interfere in the maturation process of haemopoietic cells.

Protamine sulphate, too, has been tested in view of the fact that this substance, having an anti-heparinic effect, may be substituted for embryonic extracts in the coagulation of heparinized plasma. The results of these investigations (see Astaldi, 1951) have not shown any effect of that drug on erythroblast maturation.

Proliferation

Another of the problems which invited investigations by the *in vitro* culture method, and which was included in the research programme in this laboratory, deals with the proliferative activity of the haemopoietic cells.

As is known, the microscopical observation of both histological sections and smears of bone marrow shows whether there are many or few mitoses. It is also possible to obtain the mitotic

depend on the utilization of a sufficient quantity of P.B.F., continuously carried into place by the blood stream, even in reduced concentration. Finally, it is probable that some cases of megaloblastic anaemia unresponsive to vitamin B_{12} arise from a more serious lack of P.B.F.

Going further on, we have suggested that the lessening of P.B.F. in pernicious anaemia might be a consequence of the insufficient absorption of vitamin B_{12}. In particular (see Fig. 2), the

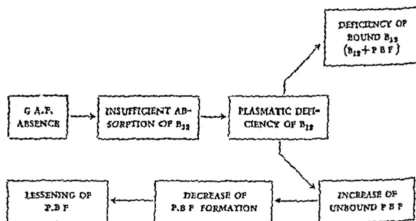


FIG 2. The relations between gastric absorption factor (G A F) absence and P.B.F.-lessening in pernicious anaemia (according to Astaldi and Cardinali, 1957)

lack of the gastric absorption factor causes a plasmatic deficiency of B_{12}, which normally gets bound to P.B.F. This induces a transitory increase of the unbound P.B.F., in comparison with that bound to vitamin B_{12}. This in turn causes a decreased formation of P.B.F., and then a more or less serious lack of it.

Besides haemopoietic factors and normal or thalassaemic or pernicious anaemia serum, other agents, such as spleen extracts, cytostatic agents, temperature, etc, have been used in experiments on the maturation of erythroblasts surviving *in vitro*.

proliferating, although at a different rate according to the cell type; second, the stathmokinetic effect of colchicine is exactly the same *in vitro* as it is *in vivo*, i.e. at the optimal dose it stops at premetaphase all the cells which have entered mitosis. In other words, the idea we have been holding is the following (Fig. 3):

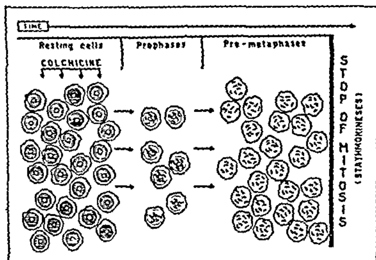


FIG. 3. Diagram of the stathmokinetic test (according to Astaldi and Mauri, 1949).

when in a given place, that is in a given culture, all the cells entering mitosis are arrested at the premetaphase, the ratio of mitoses achieved in a given time (*stathmokinetic index*) will derive just from the number of the cells which have entered mitosis, i.e. from the average duration of interkinesis. In this condition, the stathmokinetic index will show the degree of proliferation of the cells under examination.

Preliminary experiments were in agreement with the theoretical expectations, therefore the stathmokinetic test has been formulated (Astaldi and Mauri, 1949, 1950) and then used in our laboratory, as well as by other investigators.

index, that is, the percentage ratio of the cells at the mitotic stage as compared to the cells at the resting stage. But that index is not sufficient by itself to give grounds for an unequivocal conclusion on the amount of proliferation of the cells under examination. In fact, the variations of the mitotic index may depend not only on the variations in the resting time (that is, on the degree of the proliferation activity), but also on the duration of mitosis.

In other words when, for instance under pathological or experimental conditions, we observe a mitotic index which is higher than the standard one, we cannot say that more cells have entered mitosis in the same time, which would mean that proliferation was more active. In fact, the mitotic index might also be higher than the standard one because the duration of mitosis is longer than usual. The latter occurrence is not just a theoretical possibility, but a real one, because the development of the mitotic stage may be influenced by many factors, both physical and chemical, as well as under different biological conditions (see Astaldi and Mauri, 1947).

In conclusion, the mitotic index derives from two factors: *the duration of resting stage* and the *duration of mitosis*, both varying according to the cell type, the maturation stage and the different stimuli which may occur. It follows that we are not in a position to use the mitotic index to obtain information about these two factors (the one we are interested in being the average duration of the resting stage, that is, the degree of proliferation).

In order to get more precise data on which to judge the degree of cellular proliferation, we thought of arresting the process of mitosis. If this were done, the variations of the mitotic index would become a function of interkinesis only, thus expressing the degree of proliferative activity of the cells under examination as well as the effect of the stimuli eventually reaching the cells.

To do this we have experimented on the stathmokinetic effect of colchicine on cells explanted *in vitro*, based on the facts that: first, when proliferating tissues are explanted *in vitro* they keep on

between basophil and polychromatophil stathmokinetic indices is 3/1. This different behaviour of the mitotic and stathmokinetic indices as regards maturation may be explained only by admitting that mitosis lasts longer in polychromatophil than in basophil normoblasts.

From the results obtained it appears that the development of maturation in the normoblast entails: (1) a gradual increase in the

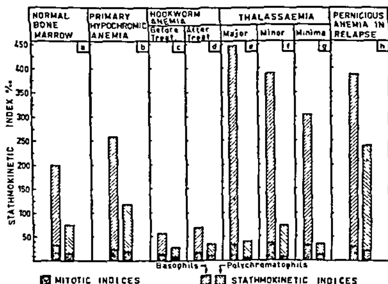


FIG. 4. Mitotic and stathmokinetic indices under different physiopathological conditions

average interkinetic time, i.e. a gradual decrease in proliferation, down to zero at the orthochromatic stage; (2) a gradual increase in the average kinetic period (mainly due to a longer metaphase).

The results of this experimental evaluation of the normoblast proliferation in the different stages of maturation agree most satisfactorily with the behaviour of the metabolism of nucleic

The test is quite simple. To the culture medium is added a solution of colchicine, whose final optimal concentration for the erythroblasts from human bone marrow is 1/1,000,000. Bone marrow fragments obtained by sternal puncture are explanted in such a medium and incubated at 37° for a given time, usually 12 to 18 hours. Afterwards, the cultures are smeared and stained according to May-Grünwald-Giemsa, or other staining methods, and at this point the number of the stathmokineses present in 1,000 cells may be determined, thus giving the stathmokinetic index.

Some results on the proliferation activity of blood-forming cells, obtained by using the stathmokinetic test, will now be discussed.

Experiments carried out on the *normoblast from normal human bone marrow* proved that (Fig 4A) the stathmokinetic index at the 18th hour of culture is roughly 200 per thousand in the basophils, and 65-70 per thousand in the polychromatophils. The stathmokinetic index of orthochromatics is practically zero, since mitoses are generally not in evidence in these cells (see Astaldi and Cardinali, 1959).

The fact that the average stathmokinetic index of the basophil normoblast is three times that of the polychromatophil one means that *the average interkinetic period of the former is three times shorter than that of the latter*, i.e. the proliferative activity of basophil normoblasts is roughly three times more than that of polychromatophil ones. The orthochromatic normoblasts, on the other hand, have no proliferative activity and therefore they have no value in the proliferation of the erythropoietic cells considered as a whole.

Information on the average duration of mitosis may be obtained by comparing the stathmokinetic indices and the mitotic ones. The latter appeared to be 31·8 per thousand ± 5·3 for basophils and 20·2 per thousand ± 1 9 for polychromatophils. The ratio of these two values is therefore 3/2, while the ratio

moderate and irregular in the minima (present in about 50 per cent of cases). It seems, moreover, that a correlation exists between clinical gravity and increased proliferation, at least within certain limits. In contrast with the behaviour of basophils, the proliferation of polychromatophils is within normal limits in all three forms of thalassaemia.

The increased proliferative activity at the basophil stage tends to offset erythrocyte losses due to the marked erythrocytolysis, while the absence of any increased proliferation at the polychromatophil stage is probably connected with the fact that no deficient maturation is seen in thalassaemia at this level.

Investigations conducted on the *megaloblast from pernicious anaemia* revealed that it proliferates more actively than the normoblast, at both the basophil and polychromatophil stages. The values of the stathmokinetic indices at the 18th hour (Fig. 411) are very high: 350-400 per thousand for the basophil and 230 per thousand for the polychromatophil megaloblast (see Astaldi and [illegible]

that while normoblast proliferation decreases from the basophil to the polychromatophil stage in a 3/1 ratio, in the megaloblast the decrease of proliferation between the two maturation stages is so much less marked that the ratio of the stathmokinetic indices is lower than 2/1. This means that the megaloblast from pernicious anaemia—in contrast to the normoblast from normal bone marrow—also proliferates actively in the polychromatophil stage

Comparison between the mitotic indices of megaloblasts (27 2 per thousand ± 1·8 for the basophil and 20·8 per thousand ± 2·2 for the polychromatophil) and the corresponding ones of normoblasts (31·8 per thousand ± 5·3 for the basophil and 20·2 per thousand ± 1·9 for the polychromatophil) does not reveal significant differences. To explain this, since the stathmokinetic

acids in normoblasts during their maturation. In fact, in the normoblast during maturation there is (Thorell, 1947) a gradual decrease of the ribopolynucleotide content, with which the protein syntheses involved in cell growth (proliferation) are connected.

Investigations on erythroblast proliferation evaluated using the stathmokinetic test have also been conducted on marrow from different physiopathological conditions.

The erythroblast from *primary hypochromic anaemia* revealed a higher degree of proliferation as compared to the normoblast from the normal bone marrow, in both the basophil and polychromatophil stages. In fact (Fig. 4B), the stathmokinetic indices at the 18th hour were 261 per thousand and 118 per thousand for basophils and polychromatophils respectively (Astaldi, Mauri and Salera, 1950). Since the mitotic indices of this anaemia are contained within normal limits, it must be admitted that the average duration of mitosis in these erythroblasts is shorter than usual. The higher degree of erythroblast proliferation, not only in the basophil stage but also in the polychromatophil one, which occurs in patients with this anaemia as compared to the normal subject, may be related to deficient maturation of the erythroblasts, depending on the lack of iron.

The opposite occurs with the erythroblast from *hookworm anaemia*, the stathmokinetic indices of which (Fig. 4C, D) are lower than normal in both the basophil and polychromatophil stages (Astaldi, Rondanelli and Troiano, 1951), despite the iron deficiency. It is therefore probable that in this anaemia the coexistence of cytotoxic damage caused by the parasite may be responsible for the decreased proliferation

The erythroblasts from *thalassaemia* showed (Astaldi and Tolentino, 1952) a proliferative activity (Fig. 4E, F, G) definitely higher than normal in all three forms of the disease: major, minor and minima. The increased proliferation is both more marked and more constant in the two more serious forms, while it is

influence of various agents on cell proliferation. It was seen that *hypoxia* is one of the conditions which inhibit the proliferation of erythroblasts. In fact, the proliferative activity (Fig. 5) decreases with the reduction of pressure in the surroundings in which the cells are cultivated (see Astaldi and Cardinali, 1959). A decrease

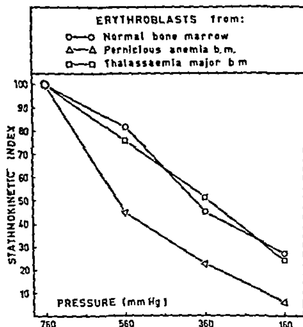


FIG. 5. Influence of hypoxia on proliferative activity of erythroblasts under different conditions.

in the erythroblastic proliferation induced by hypoxia is revealed in both the basophil and polychromatophil stages, but it is more marked in the former.

Similar investigations conducted on the megaloblast from pernicious anaemia proved that hypoxia causes a greater reduction of proliferation in this type of erythroblast than in the normoblast, and also that in megaloblasts the sensitivity to hypoxia is

indices of the megaloblasts are higher than those of the normoblasts we must admit that the average duration of the megaloblastic mitosis is shorter than that of the normoblastic one.

The following conclusions may be reached with regard to the megaloblast: (1) it proliferates much more actively than the normoblast in both the basophil and polychromatophil stages; (2) the decrease in proliferation when passing from the basophil to the polychromatophil stage is less marked than that observed in the normoblast; (3) the average duration of mitosis in megalo- ... be shorter than that in normoblasts. ... tivity of the

... oblasts

Experiments carried out on ... proved that the basophil megaloblast in a two-day-old embryo shows a very intense proliferation. In fact, its stathmokinetic index is 510 per thousand, i.e. even higher than that in the megaloblast from pernicious anaemia (400 per thousand). It is interesting to note that, when passing from the basophil to the polychromatophil stage, proliferation decreases much more in the megaloblast from the embryo than in that from pernicious anaemia. In other words, as regards relationship with the maturation stage, we note that the behaviour of the embryonic megaloblast's proliferation is more similar to the normoblast's than to that of the megaloblast from pernicious anaemia. This behaviour apparently confirms the concept that no biological identity between the embryonic megaloblast and the pernicious anaemia megaloblast exists.

With regard to the embryonic erythroblast from the second erythropoietic series—the normoblastic—it was observed that its proliferative activity is much lower than that in the first series—the megaloblastic. Moreover, it was observed that proliferation decreases when passing from the basophil to the polychromatophil stage.

A number of experiments were carried out to investigate the

erythropoietin is present within the erythroblasts of the anaemic animal, or closely attached to them, so that it exerts its mitotic stimulating effect within the cells.

Investigations have also been conducted to test the influence of *X-rays* on the proliferative activity of surviving erythroblasts from human bone marrow, i.e. on both normoblasts from

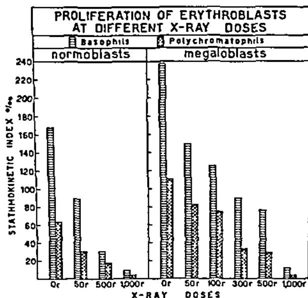


FIG. 6. Influence of X-rays on proliferative activity in both normoblasts and megaloblasts.

normal subjects and megaloblasts from pernicious anaemia patients in relapse. During these experiments, bone marrow cultures were subjected to X-ray doses ranging from 50 to 1,000 r. Fig. 6 illustrates the average stathmokinetic indices obtained after 14 hours of culture both in the controls (0 r.) and in the irradiated cultures. On the left are seen the results for both the basophilic and the polychromatic normoblasts (see Astaldi

more marked in the basophil than in the polychromatophil stage. It is possible to note that the different sensitivity of megaloblasts and normoblasts to the deficiency of O_2 is gradually reduced by the effect of the specific treatment.

It appeared that the greater sensitivity to hypoxia of the megaloblasts as compared to the normoblasts is not simply the consequence of the fact that the former has a higher proliferative activity than the latter. Indeed the erythroblasts from thalassaemia major, which proliferate nearly as actively as the megaloblasts, behave like the normoblasts with respect to hypoxia. We are led to the conclusion that the more marked sensitivity to hypoxia shown by the megaloblast is correlated with the metabolism peculiar to this cell.

Another agent which appears to be inhibiting proliferation is *vitamin B_{12}* (Astaldi and Strosselli, 1959). In fact, the determination of the stathmokinetic index in the migration area of fibroblast cultures showed the indices to be lower than in the controls when vitamin B_{12} is present. In particular, indices just half those of the controls appeared when the vitamin contents ranged from 10 to 1 μg/ml., and they gradually increased as the vitamin concentration was reduced, until a point was reached where, when the dosage was about 0 0001 μg/ml., no significant difference existed as compared to the controls.

Matoth and Edna Ben-Porath (1959) carried on researches, using the stathmokinetic test, in order to test the influence of *erythropoietin* on the erythroblast proliferation. These experiments showed an increase of erythroblast proliferation when the marrow was cultured in a medium containing serum from rabbits made anaemic by bleeding Moreover, it appeared that the erythroblasts from rabbits made anaemic by bleeding proliferate at a higher degree (400–500 stathmokineses in 1,000 cells) when growing in both normal and anaemic serum. The authors conclude that these results provide evidence for a mitotic stimulation by erythropoietin at the bone marrow cellular level, and that the

polychromatophil ones. It is possible that such a difference may be connected with the different degree of proliferative activity in these two phases of maturation (higher in the basophil than in the polychromatophil stage), which in its turn is a consequence of the biochemical differences between these two phases (ribo-

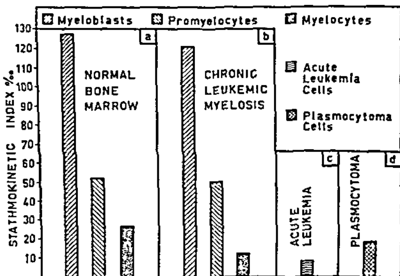


FIG. 7. Stathmokinetic indices of white cells and plasmocytoma cells in the normal and in leukaemias

and deoxyribonucleic acid, protein, haemoglobin, enzyme, etc., contents).

Observations on the *leucoblasts from normal subjects* revealed that in these cells also, proliferation gradually decreases with the progress of maturation (Fig. 7A). In fact, the stathmokinetic indices of leucoblasts determined in normal bone marrow cultures at the 18th hour are 128·1 per thousand ± 4·29 for myeloblasts, 52·60 per thousand ± 2·29 for promyelocytes, and 26·90 per thousand ± 1·43 for myelocytes (Salera and Tamburino,

and Cardinali, 1959; Astaldi, 1958). On the right are the results of the röntgen irradiation on cultures of bone marrow from pernicious anaemia in relapse (Astaldi, Strosselli and Karanovic, unpublished data).

First of all, confirmation may be obtained that megaloblasts proliferate at a higher degree than normoblasts, the controls of the former having a stathmokinetic index (after 14 hours of culture) of 240 per thousand in the basophil stage and 110 per thousand in the polychromatic one, while the corresponding indices of normoblasts are 140 per thousand and 65 per thousand, respectively. Moreover, it may be observed that the proliferation of both normoblasts and megaloblasts is very sensitive to X-ray irradiation, already appearing markedly inhibited with 50 r. Finally, such findings show that the extent of proliferative inhibition depends on the X-ray dose employed, but not proportionally.

If the percentage decrease in proliferation exerted by the different doses of X-rays on the normoblast and the megaloblast (at the basophil stage) is compared, it can be found that, with 50 r., the reduction in proliferative activity is about 50 per cent for normoblasts and only 37 per cent for megaloblasts; with 500 r., it is about 80 per cent in the former and 70 per cent in the latter, finally, it becomes nearly 100 per cent with 1,000 r. In conclusion, it appears that proliferation in both normoblasts and megaloblasts is very sensitive to the action of X-rays, but to a different degree. Workers in this laboratory believe that this difference between normoblasts and megaloblasts depends on the peculiar characteristics directly connected with the different biology of these two types of erythroblasts

With reference to the maturation stage, it appeared that the basophil and the polychromatophil normoblasts show a different reactivity under the anti-mitotic action exerted by X-rays On the whole, it would seem that the proliferating activity of the basophil erythroblasts is more radically inhibited than that of the

much more marked in the leukaemic granuloblasts than in the normal ones.

On the basis of the aforementioned data, the following conclusions may be reached: (1) leukaemic granuloblasts, considered as a whole, do not proliferate more actively than the normal granuloblasts, in either the myeloblast or promyelocyte stages; (2) the decrease of proliferation in the myelocyte stage is more marked in leukaemic cells than in normal ones. This latter fact would seem to indicate that, while in the early maturation stages the proliferative activity of leukaemic cells is not especially affected, in the more advanced stage such activity is more seriously affected, probably on account of difficulties intimately connected with the maturation process.

The observations of proliferative activity by use of the stathmokinetic test proved that the haemocytoblast from *acute leukaemia* is a cell that scarcely proliferates; in fact, its stathmokinetic index (Fig 7C) is roughly 7 8 per thousand at the 14th hour of culture, according to Astaldi and Mauri (see Astaldi and Cardinali, 1959). Similar results were likewise obtained by Sacchetti and Bianchini (see Astaldi and Cardinali, 1959) and by Salera and Tamburino (1953). These data may be considered as a strong objection to the hyperplastic theory of the genesis of this leukaemia.

The results obtained in cases of *plasmocytoma* proved that in the plasma cell series too, the behaviour of the proliferative activity in relation to the maturative evolution is similar to that observed in both the erythroblast and granuloblast series. The stathmokinetic index in the mature plasmocyte type is rather low, roughly 4 to 5 per thousand, thus proving that the mature plasma cell has a low proliferative activity. In forms of plasmocytoma with immature cells of a mainly plasmoblastic type, the stathmokinetic index (Fig 7D) is 16 to 18 per thousand, i.e. from three to four and a half times higher than in mature cell forms (see Astaldi and Cardinali, 1959).

1953). Therefore, the proliferative activity in myeloblasts appears to be approximately five times more than that in myelocytes and two and a half times more than that in promyelocytes, which in turn, proliferate twice as much as myelocytes.

The ratio between the stathmokinetic indices of myeloblasts and those of promyelocytes corresponds to the ratio between the respective mitotic indices, which are apparently 31·3 per thousand and 13·7 per thousand in myeloblasts and promyelocytes respectively. This fact would prove that no important differences in the duration of mitosis exist between myeloblasts and promyelocytes. However, the ratio between the stathmokinetic index of myeloblasts and that of myelocytes is definitely higher than the ratio between the respective mitotic indices, which are 31·3 per thousand for myeloblasts and 8·8 per thousand for myelocytes, i.e. a 3·6 ratio. This would indicate that the average duration of mitosis is longer in myelocytes than in myeloblasts.

We may conclude, therefore, that leucoblasts behave like erythroblasts as regards the relationship between proliferation and maturation. As a matter of fact, in granuloblasts we also note that with the progress of maturation the proliferative activity decreases, i e the average interkinetic time increases. Moreover, in passing from the myeloblast to the myelocyte stage, the average kinetic time increases, mainly on account of the lengthening of the metaphase.

The stathmokinetic indices in *chronic leukaemic myelosis* at the 18th hour of culture (Fig. 7B) are 120·5 per thousand for myeloblasts, 49 per thousand for promyelocytes, and 11·8 per thousand for myelocytes (Salera and Tamburino, 1953). A comparison between the stathmokinetic indices of leukaemic granuloblasts with those of normal ones shows that the former are lower than the latter. While the differences are not significant in the myeloblast and promyelocyte stages, they are definitely so as regards the myelocyte stage. It follows that in passing from the promyelocyte to the myelocyte stage, the decrease in proliferation is

Differentiation

Finally, some results regarding the differentiation process, as examined by the tissue culture method, will be discussed. On this subject, studies in this laboratory are less numerous and still at a preliminary stage as compared with those researches which deal with maturation and proliferation processes of the haemopoietic cells. However, the results obtained with vitamin B_{12} and with a bone marrow extract are submitted here for criticism.

The first series of experiments was performed (Astaldi and Strosselli, 1959) on fibroblasts isolated from myocardial fragments of chick embryos at the seventh day of development. These cells were cultivated *in vitro* in a fluid medium ("199 mixture", Microbiology Association, Bethesda, Md.), both with and without *vitamin* B_{12}. The plane supporting cell migration was the surface of the slide on which the small fragments of embryonal myocardium were applied. By this method the introduction of any other biological factor into the standard medium used (199 mixture) has been avoided, which would not have been possible if the cell substratum had been providing the usual plasma clot. In addition to this, the cells were compelled to migrate over a single-layer surface which favourably affects and makes uniform the nutritional exchanges between the cells and the medium, while also making a microscopical examination of the cells considerably easier.

Vitamin B_{12} (Farmitalia, Milano, Italy) was used in doses of 10, 1·0, 0·1, 0·01, 0·001 and 0·0001 μg./ml. of 199 mixture. Cultures prepared from these media, as well as the control ones without vitamin B_{12}, were kept in roller tubes at 37·5°. In the cultures containing the largest doses of vitamin B_{12} (10, 1·0, 0·1 μg./ml.), the presence has been noticed of some cells with a marked increase in their cytoplasmic basophilia, related to an increase in the ribonucleotide thickening patterns. In these cells,

As regards the mitotic indices, they are lower both in the immature and mature cell forms. They generally range from 0·1 to 2·7 per thousand. Out of ten cases, higher values have been found in one case only. It follows that, if the stathmokinetic indices are compared with the respective mitotic ones, in plasmocytomas of the immature type the stathmokinetic index is 3 to 17 times higher, and in mature cell plasmocytomas it is only three to four times higher. This means that when passing from the plasmoblast to the plasmocyte not only does the average interkinetic time increase (i.e. the proliferative activity decreases), but the average kinetic time also increases.

On the basis of all the foregoing results, Astaldi and Cardinali (1959) drew up the following principles of bone marrow physiopathology:

(1) The proliferative activity of blood-forming cells varies with the modification of the cell type (erythroblast, granuloblast, plasmocyte).
(2) A relationship exists between proliferative activity and maturation stage that in normal conditions is constant for each cell series.
(3) Independently of the cell series, with the development of maturation a gradual decrease of proliferation occurs, i.e. an increase in the average interkinetic time.
(4) With the development of maturation, it is possible also to observe an increase in the average kinetic time. This increase generally depends on the lengthening of the metaphase.
(5) The more immature cells, i.e. the more actively proliferating ones, are more sensitive to the effects of factors inhibiting proliferation.
(6) The decrease in the proliferative activity when passing from the more immature to the more mature stages may likewise be observed in pathological conditions (anaemia, leukaemia, plasmocytoma).

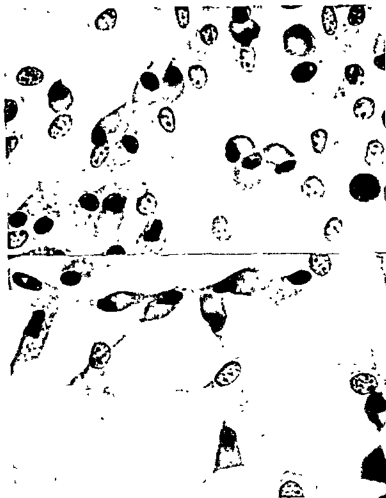


Fig 8 Plasma cell differentiation *in vitro* caused in previously undifferentiated cells by the action of a bone marrow extract

facing page 1

moreover, the chromatin appeared much coarser than in the unchanged fibroblasts. Finally, these cells appeared more regular in their surroundings so that, at the end of all the modifications mentioned, these cells showed the same findings as in the embryonal promegaloblasts.

From the second series of culture experiments, those where a *bone marrow* extract had been added to the culture medium, plasma cells from primitive embryonic histoid cells seem to have been obtained.

To test this, myocardial fragments from chick embryo at the eighth day of development have been explanted (Strosselli and Astaldi, 1960) in the following medium: Eagle Basal Medium (Microbiology Association, Bethesda, Md.) 90 ml, horse serum 10 ml., and (except in the controls) the bone marrow extract (prepared by Robapharm Labs., Basel, Switzerland).

In the untreated cultures, the usual histoid-fibroblastic strains have been obtained. In the cultures with the bone marrow extract, from the tenth day, it is possible to observe that some cells, probably deriving from the primitive endocardium, undergo marked morphological modifications during development (Fig. 8). Their nuclear chromatin pattern becomes thicker, so that the whole nucleus appears more coloured, while the cytoplasm becomes much more basophilic. Meanwhile, in the cytoplasm some vacuoles appear which are grouped in a paranuclear position, definitely like those which are contained in plasma cells. In fact, the strong basophilia of the cytoplasm surrounding these vacuoles leads one to believe they are of an ergastoplasmic nature. Finally, the nucleus assumes a position beside the centre, while the entire cell becomes ovoidal These cells therefore show typical plasmocytic findings.

These results seem to confirm the theory of the reticular genesis of the plasma cells. In addition, they show the existence of connexions between the differentiation process and the development of the cells from the primary syncytium. Further investiga-

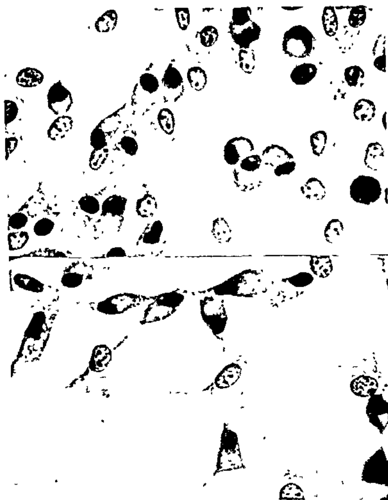


FIG. 8 Plasma cell differentiation *in vitro* caused in previously undifferentiated cells by the action of a bone marrow extract

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tions on this subject of cellular differentiation *in vitro* are included in our programme.

Summary

Maturation of haemopoietic cells is studied by maintaining medullary cells in a liquid medium and every 6 to 12 hours counting the number of cells at the different phases of maturation. Since under these conditions haemopoiesis rapidly diminishes while maturation proceeds, the first cells to decrease in number or disappear are the most immature ones, then those in an intermediate stage and, finally, those which are close to maturity. The length of life can be estimated from this behaviour of the cells.

Erythroblasts have been studied in both normal and pathological conditions, e.g. pernicious anaemia in relapse, primary hypochromic anaemia, thalassaemia (major, minor and minima), etc. Moreover, experimental work has been carried out on the effects of various agents on maturation of erythroblasts, e.g. temperature, colchicine, liver extract, vitamin B_{12}, nicotinamide and the effect of normal serum on pathological erythroblasts, etc.

Proliferation is investigated on fragments of bone marrow explanted into a solid medium containing colchicine at optimal concentration. Owing to the stathmokinetic action of colchicine, all the cells entering the mitotic stage are arrested at the premetaphasic stage, the result being that the number of mitoses in the culture (or stathmokinetic index) gradually increases with time. The amount of proliferation can thus be deduced from the increase in the number of stathmokineses reached at a given time

Studies on the proliferative activity have been carried out with both erythroblasts and leucoblasts under different physiopathological conditions, e.g. erythroblasts from normal bone marrow and from primary hypochromic anaemia, hookworm, anaemia, thalassaemia, pernicious anaemia in relapse, on embryonic

erythroblasts, and on leucoblasts from acute and chronic leukaemic myelosis and, finally, on plasmocytoma cells. Moreover, the effects of ionizing radiations, hypoxia and other agents on the proliferative activity of haemopoietic cells have been investigated.

Research into differentiation is not yet far advanced. Using vitamin B_{12} it seemed possible to obtain differentiation from primitive embryonic mesoblasts to promegaloblasts. Moreover, using a bone marrow extract on primitive embryonic histoid cells, plasma cells have been obtained. Further investigations of this subject are in progress.

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DISCUSSION

Rachmilewitz: How do you explain the difference resulting from the addition to the serum medium of B_{12} and B_{12} plus gastric juice?

Astaldi. I have answered this question in the printed paper, Prof. Rachmilewitz. I might add here that by comparing both the *in vitro* and *in vivo* observations it is possible to deduce that in pernicious anaemia there is a lessening of a plasmatic factor, or *utilization factor*, which is needed for the utilization of vitamin B_{12} by blood cells. One may suggest that such a plasmatic factor may be the plasmatic binding factor (P.B.F.), whose lessening may explain the lack of vitamin B_{12} action *in vitro*, where there is a very small quantity of serum. Such a defect is obviated by adding protein substances contained in embryo extract, or in gastric juice. However, *in vivo* vitamin B_{12} may become biologically active, because there is not an absolute deficiency of P.B.F. owing to the great quantity of serum present in the entire body, although the percentage of P.B.F. is less in this serum.

Rachmilewitz: Your explanation is very interesting, but experiments on the binding capacity of sera of pernicious anaemia have not shown any difference from the normal.

Lajtha: Binding and binding are two different things. F. L. Ternberg and R. E. Eakin (1949. *J. Amer. chem. Soc.*, 71, 3858) have found that saliva will bind B_{12}, but it is known that this particular binding has no intrinsic factor activity. In 1954 we found we had to differentiate very strongly between binding and intrinsic factor activity. We returned then to the original Castle hypothesis, that haemopoietic factor equals extrinsic plus intrinsic factor. Intrinsic factor then is not merely an

absorption factor but a true addition to B_{12} (the extrinsic factor). When we add normal gastric juice to our cultures we are adding active intrinsic factor which can be destroyed by heating, without destroying B_{12}-binding capacity.

Astaldi: On this question, Prof. Rachmilewitz, I should like to draw attention to the results obtained by Horrigan and Heinle (1952, *loc. cit.*), as well as those of Pitney, Beard and Van Loon (1954, *loc. cit.*). They showed that vitamin B_{12} is contained in normal human serum in a bound form, and also that in normal conditions the bound form of vitamin B_{12} is chiefly contained in the α-globulin fraction of human serum.

Gordon: Dr. Matoth in Jerusalem has recently made the interesting observation that when marrow from anaemic animals was incubated with normal serum in his colchicine-plasma clot system, high mitotic rates among the erythroblasts were noted He suggested that this is indicative of the presence of erythropoietin in the erythroid elements I believe it was you, Dr. Astaldi, who demonstrated the presence of PAS (periodic acid-Schiff)-positive material in the cytoplasm of erythroblasts from thalassaemia major subjects. In view of Matoth's findings, and since erythropoetin appears to be a mucoprotein, do you think there may be some connexion between these inclusions and the active principle?

Astaldi: My results agree completely with the results you have mentioned, Dr. Gordon. In fact, by 1952 (Astaldi and Tolentino, *loc. cit*), we had observed that the bone marrow erythroblasts from thalassaemia patients proliferate at the same level when the marrow is cultured both in the serum from the same thalassaemic donor of the marrow, and in the serum from a normal subject. That is, thalassaemia erythroblasts in normal serum may proliferate twice as actively, or even more, as normal bone marrow erythroblasts.

Regarding the periodic acid-Schiff-positive abnormal substance present in the erythroblasts from thalassaemia major you have kindly mentioned, according to the results of our cytochemical investigations (Astaldi, G., Rondanelli, E G., Bernardelli, E , and Strosselli, E. [1954]. *Acta haemat. (Basel)*, **12**, 145), it seems to be a gluco- or a mucoprotein, or possibly a neutral mucopolysaccharide, but it seems difficult to admit the existence of correlations between erythropoietin and the

above-mentioned PAS-positive structures. In fact, until now we have not been able to show the same structures in the erythroblasts from patients affected with other anaemias, where the erythropoietin content is high, since PAS-positive granules have been observed in the erythroblasts from a few other conditions, such as leukaemias treated with antimitotic agents, benzene intoxication, etc. In fact, the histochemical tests showed a very different structure between these latter PAS-positive granules and those in the thalassaemia erythroblasts. Together with Dr. E. Strosselli, I have observed structures which previously seemed to be analogous to those in thalassaemic erythroblasts; this was in a patient met last summer in Chile that Prof. Wintrobe described as a case of pyridoxine-deficient anaemia. Perhaps we can be more precise on this latter subject when all the cytochemical investigations under way have been carried out.

Fichtelius: The cells in your Fig. 8 seem more like osteoblasts than plasma cells to me, Dr. Astaldi. In plasma cells the light zone, the archoplasm, is perinuclear; in osteoblasts the light zone is located peripherally as in your cells.

Astaldi: Those are the cells we have obtained culturing a chick embryo myocardial fragment in the presence of a bone marrow extract. They seem very similar to the plasma cells of chickens, and many of them, though not all of them, show that the light zone is located perinuclearly.

Jacobson: What ultimately happens to this culture with these cells as they live on?

Astaldi: The cells which I have shown as the most mature plasma cells are the final stage of the observed differentiation.

Lajtha: May I suggest that you test the uptake of [^{35}S]sodium sulphate? If these are plasma cells they should not show any uptake, and if they are osteoid in nature they may well show quite significant uptake.

Astaldi: Thank you, Dr. Lajtha; I think that your suggestion is a good one. Dr. M. Bessis said last May that he also believed that these cells are plasma cells from the morphological point of view, and others agreed with this concept. But I would like to consider also the concept that Dr Fichtelius has mentioned, and leave open the possibility that further morphological, biological or biochemical

investigations may prove that those cells are osteoblasts, or other cells In any case, it is important to stress that they are differentiated cells, as compared to the histoid fibroblast strain from where they started.

Cronkite: I have no good idea what these cells are, but a similar problem has arisen as to what types of cell are actually coming out of the thoracic duct. By phase microscopy with living cells, and staining, we have not seen typical plasma cells or their precursors, monocytes, or phagocytic cells, as yet. In discussion of this with Dr. Bessis a year ago it seemed a good idea to look at these in the electron microscope. It was quite a jolt to me while working with him last week to see that there are monocytes in canine lymph as well as plasma cell precursors that we had overlooked by phase microscopy and ordinary staining procedures.

Yoffey: The plasma cell precursor has been known for some little time from the work of T. Wesslén (1952. *Acta derm.-venereol. (Stockh.)*, 32, 265). He gave his animals injections of living typhoid bacilli, then showed there was no antibody in the thoracic duct lymph; but if he cultivated it in roller tubes he got antibody formation. It has been made clear from that type of observation, and also by inference from the subsequent accumulation of actual plasma cells in the lungs—the first place where they could be trapped in a capillary bed and be held up—that there are precursors of antibody-forming cells in lymph But I did not realize that they were already showing the typical plasma cell structure which Dr. Braunsteiner and his colleagues showed some years ago (Braunsteiner, H , Fellinger, K. and Pakesch, F. [1953]. *Blood*, 8, 916).

Cronkite: They are very easy to find. We are perhaps having a difficult enough time in understanding the normal cells However, I was interested in the low stathmokinetic index in plasmocytoma and acute leukaemia, which does not seem to be consistent with rapid cell turnover.

Astaldi: In a given cell population, it seems to me important to consider separately the average proliferation activity of that population, and the degree of proliferation of those cells which are proliferating. For instance, in haemocytoblastic acute leukaemia all the haemocytoblasts seem to be identical from the morphological point of view, but obviously it is not so from the chronological point of view. In fact,

while haemocytoblasts in normal bone marrow develop into myelocytes and perhaps also into erythroblasts in a few hours, haemocytoblasts in acute leukaemia complete their biological cycle in the haemocytoblastic stage. Thus, while in normal bone marrow you deal only with very young haemocytoblasts, in acute leukaemia you deal with haemocytoblasts few of which are young and most of which are old cells. Thus, in acute leukaemia the proliferative activity of these cells, on an average, is low, and the stathmokinetic test shows only the average proliferation.

CELL TRANSFUSION AND ITS SIGNIFICANCE IN RELATION TO BLOOD CELL FORMATION

J. F. Loutit

M.R.C. Radiobiological Research Unit, Harwell, Berks.

Our basic knowledge of haematopoiesis has been derived from the use of the light microscope. Today any good textbook of histology will give in words and pictures the distilled essence of a century's work of patient observation and logical deduction by many of the greatest names in the history of biology. But like any single method of investigation conventional histology has its limitations, in this case the physical limitations of the light microscope and the added limitation of interpreting a dynamic process from a somewhat random number of static events. We may expect to overcome some of the deficiencies by means of the electron microscope (see Bessis, 1954) and some by the use of techniques other than the purely histological. The complementary method of investigating haemopoietic tissue, through sampling by needle-puncture and examining stained smears of the cellular products, has been utilized for decades To this can now be added the devices of labelling certain cells supravitally, e.g. by fluorescent dyes or radioactive tracers (see Everett *et al.*, 1960; Cronkite *et al.*, 1960), to follow the distribution of the label and its relations to time.

These exercises can be undertaken in the functionally normal subject. However, it is a long and well-established principle that lessons in physiology can also be learnt from study of the disturbed function in pathological states. From such pathology we deduce that the bone marrow, the normal site for production of

red corpuscles, granulocytes and platelets, can be considered as a tripotent tissue, "erythron", "leucon" and "thrombon". Each element can be congenitally defective or missing and each can be stimulated to hyperplasia, depressed to hypoplasia and even aplasia, or disturbed (dysplasia). Depressants having been removed or nutritional deficiencies made good, one can then observe the processes of recovery from hypoplasia or change from dysplasia. This is especially helpful in the understanding of normal haematopoiesis.

In the experimental laboratory one of the most powerful depressants of active haemopoiesis is exposure of the animal to

tages of such radiations are that, when they are given at suitably high intensity, the result can be limited to a very short period of time, if need be to the almost instantaneous. Thus the consequent phases of destruction and repair can be related strictly to the time of injury. Furthermore the dose can be graded to any desired amount and can be accurately defined. One disadvantage is that the secondary effects due to damage of other radiosensitive tissues and the general interactions of the integrated organism do pose complications, but this type of demerit applies to other forms of damaging agent also.

Two substantial contributions to our knowledge of the qualitative effects of such injury and its repair have recently been made At the Radiobiological Research Unit Hulse (1959) has used the albino rat, and in Bristol Harris (1960) has used the guinea pig. Hulse, like Bloom (1948) earlier, has confirmed the extreme radiosensitivity of the erythron. The degenerative changes were the earliest and most profound in this cell series and its recovery in the rat also preceded that of the other cell populations. The most striking of Hulse's findings was the apparently greater sensitivity of the more mature than the earlier normoblasts. This

could be, according to his interpretation, an acceleration of normal degenerative changes of the nucleus and its extrusion. The granulocytic series, while less reactive to the radiation injury, was much slower to recover than the erythron and this slow climb towards the pre-irradiation values was interrupted. Thus Hulse has seen what had previously impressed workers in Chicago (e g. Jacobson *et al.*, 1954) with other species—the early abortive rise. Abortive rises were also noted by Hulse in the megakaryocytic series. The lymphocytes of the bone marrow as in the peripheral blood responded rapidly with a fall in numbers. The total lymphocyte count in marrow, however, was restored to normal comparatively quickly, whereas the count in peripheral blood remained much less than normal throughout the period of study. Harris on the other hand has confirmed (1960) his earlier published observations (1956) that, in the guinea pig following a dose of 150–170 r. of ^{60}Co γ-rays, recovery of the bone marrow is preceded by the appearance in the marrow of cells which appear to be small lymphocytes and larger cells transitional between these lymphocytes and blast cells. This led him to the suggestion, following Yoffey and Courtice (1956), that small lymphocytes could be considered as the precursors by a dedifferentiation through transitional forms to blast cells, presumably multipotent haemocytoblasts. An alternative hypothesis, that the lymphocytes may have accumulated as donors of deoxyribonucleic acids (Hamilton, 1956) or the like, was also considered.

Had the transformation of the small lymphocyte to haemocytoblast been an essential step in normal mammalian haemopoiesis and repair of radiation-induced aplasia of the marrow, one would expect to find Harris's phenomenon of prehaemopoietic accumulation of lymphocytes in other species. However, in his extensive studies of rats irradiated with the various doses—100, 200 and 400 r.—of X-rays Hulse did not see it. Trowell and the present author (unpublished) did examine at the recommended time the bone marrow of several of the Radiobiological

Research Unit's stock of guinea pigs irradiated with 150 r. of X-rays: we confirmed the accumulation of small round cells and were satisfied that they were small lymphocytes. Thus the phenomenon is not unique to Harris's stock and environment. It is more likely to be a phenomenon peculiar to guinea pigs.

The rôle of the lymphocyte in bone marrow therefore still requires elucidation. That they are normally present in enormous numbers, as noted by Yoffey (Yoffey and Courtice, 1956), is without question. Are they merely sequestered there to provide a reserve pool for the peripheral blood? This seems unlikely, since Hulse found a chronic lack of lymphocytes in the peripheral blood for weeks after the numbers in marrow had been restored to normal following irradiation. Are they there to donate, either alive or dead, their substance to other cells? This is compatible with the data so far Since labelled lymphocytes seem to be lost from the lymph tissue and circulation in a random exponential fashion (Schooley, Bryant and Kelly, 1959), and since they accumulate with time in marrow, the findings could be interpreted as showing that this is their final resting place. Nevertheless their presence does not seem indispensable (see below).

The evidence from experimental work with irradiation so far presented has been confined to sublethal irradiation. Recovery then takes place from primitive cells which by chance have not suffered lethal damage, but which nevertheless must have absorbed energy in non-vital parts. It is now possible by the arts of the laboratory to study haemopoiesis after lethal doses of irradiation. All primitive haemopoietic cells are then destroyed or so severely damaged that they cannot compete with an injected suspension of haemopoietic cells drawn from a normal animal. This is given as restorative therapy. The normal haemopoietic cells, administered intravascularly, "home" to haemopoietic tissue and recolonize it

The suspensions shown to be therapeutically effective in this respect are of bone marrow of the adult animal, of spleen in the

case of young mice where the spenic pulp is haemopoietic, and of foetal liver. Most of the experimental work has been done with mice, but rats, rabbits, dogs, monkeys and man have also provided some data. It has been found profitable so far to concentrate on the mouse for immunological reasons. The acceptance of a functioning graft, which fundamentally is the case in this situation, depends on the inactivation by the heavy dose of radiation of the host's normal immunological responses to grafts of foreign tissue (usually homografts). This inactivation may be only temporary. In such a case it is desirable to work within inbred strains which are virtually all isogenic. Only mice and rats provide the necessary experimental animals. However, when host and donor are genetically identical, no genetically determined markers are ordinarily available for differentiating the products of host and donor. Working between strains of different constitution does provide markers but involves the immunological complications of possible reactions of host-versus-graft and graft-versus-host. Since so much more is known about the genetic constitution of mice than other mammals, once again it is advantageous to use the mouse as the experimental animal.

Our own experiences (Ford *et al.*, 1956; Ford, Ilbery and Loutit, 1957) with such foreign grafts of normal haemopoietic tissue have involved chromosomal markers. Our usual procedure has been to give our inbred, pure strain CBA mice, which have the normal murine chromosomal complement, 950–1,000 rads ($\sim LD_{98}$) of X-rays This is followed by an intravenous injection of a suspension of haemopoietic cells, either from the rat, which has chromosomes which differ morphologically and numerically from those of the mouse, or from a non-inbred line of mice which we call T6 (Carter, Lyon and Phillips, 1955). This line has [illegible] whom a chromosome trans- [illegible] ie of the translocated chromo- [illegible] c examination of cells at the metaphase of mitosis.

The results of the experimental work showed that the CBA mice, during recovery from the irradiation, had 100 per cent of their mitotic cells in bone marrow and spleen of the donor's chromosomal type. Furthermore, the findings in the purely lymphoid tissues of lymph node and thymus were the same. One explanation of this would be that the injected suspension contained multipotent cells which could differentiate into myeloid* tissue if they settled in bone marrow or splenic pulp and into lymphoid cells when they colonized lymphatic tissue. Alternatively the injected material included both marrow-forming and lymph tissue-forming precursors. This latter could understandably be the case when the reconstituting suspension was made from juvenile spleen, which is accepted as being composed of white lymphoid follicles and red myeloid pulp. Bone marrow from the adult mouse is usually credited, however, with myeloid activity only. While lymphocytes are present in the bone marrow in considerable numbers apparently distributed at random, it is usually stated that there is no organized lymphoid tissue in marrow and hence no production of lymphocytes there. Dr. O. A. Trowell, whose knowledge and experience of lymphatic cells and tissue is without equal, has convinced the present author in discussion that such negative evidence is of little value and that there is no fundamental objection to formation of lymphocytes in this diffuse fashion.

For the time being let us assume that some of the blast cells in marrow are lymphoblasts. Do they arise from basic reticulo-endothelium there, or do they come from small lymphocytes carried there by the blood stream and entering the parenchyma from the sinuses, to dedifferentiate through transitional forms to blast cells as envisaged by Yoffey and Courtice (1956) and Harris (1960)? We know of no convincing evidence for one or the

* In this paper the term "myeloid" is used in the primary sense of "marrow-like", that is, productive of red corpuscles, platelets and granulocytes; it is not used, as it is by others, as a synonym for "granulopoietic".

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When foreign haemopoietic tissue is given as therapy, the regeneration of myeloid tissue is just as satisfactory but the lymphoid tissue may never satisfactorily recover. The chimerical animal after surviving the period of the radiation syndrome may manifest a "secondary disease" which is often lethal. It is associated with profound loss of condition and wasting, often with diarrhoea (depending on the variety of the host), and histologically is characterized by atrophy and hyaline changes in lymphatic tissue (Congdon and Urso, 1957). We have attributed this (Barnes *et al.*, 1957) to a graft-versus-host reaction, the antigenic material of the host being conveyed by the normal physiological routes, the lymphatics, to the local lymph glands where it meets graft-type activated lymphoid cells to react in allergic fashion involving mutual destruction. Characteristically the marrow is unaffected and histologically normal even in fatal cases of the "secondary disease" (Barnes *et al.*, 1958). This is one of the pieces of evidence which makes us believe the precipitating factor in the "secondary disease" is a graft-versus-host rather than a host-versus-graft reaction.

If we now consider the suggestion raised above that the marrow as well as lymphatic tissue is actively producing lymphoid cells, why is it not as severely damaged as the organized lymphatic tissue in secondary disease? We have long recognized that the marrow actively produces plasmatoid cells (and sometimes is the seat of plasmacytomas) and can produce humoral antibodies (Askonas and White, 1956). It can thus respond to the H antigens of Medawar (1959) brought to it by the blood stream can it, however, react to transplantation T antigens which presumably would have to reach it by the same route? The Malpighian bodies of the spleen and the thymus are involved in secondary disease and presumably host's antigen cannot reach them like lymph glands by afferent lymphatics. Another look at the bone marrow in secondary disease with particular emphasis on its lymphoid cells is called for. At present the predominant impression

other. Nevertheless the preliminary observation of Schooley, Bryant and Kelly (1959), that following a single administration of tritiated thymidine as many as 50 per cent of the marrow "lymphocytes" were labelled after three days, and, following daily injections, 100 per cent were labelled as against only 10 per cent in the peripheral blood and lymph, is compatible with the marrow being a factory for production of lymphocytes as well as of the other cells of peripheral blood. Hitherto, with the impression, perhaps wrong, that the absence of lymphoid aggregates in the marrow indicated absence of production, it was suggested (Loutit, 1960), following Yoffey, that differentiation from small to large lymphocyte in the marrow might occur normally with haematogenous metastasis of the large lymphocyte to lymphatic tissue. This would explain, as would "unorganized lymphopoiesis" in marrow, why suspensions of marrow will cause immediate recolonization of the lymphatic as well as the myeloid tissues of the lethally irradiated mouse.

It is worth noting at this stage that the organized lymph tissue—lymph nodes and thymus, for example—takes much longer to be restored histologically than bone marrow and splenic pulp, when the lethally irradiated mouse is treated with haemopoietic cells. This is best seen when fully compatible isogenic cells from the same strain can be used and occurs even when known lymphoid as well as myeloid precursors are given as suspension of infant spleen (Barnes and Loutit, 1956). The regenerated marrow has occupied the available space in little more than a week and appeared mature after two: the lymphoid tissue takes many weeks to reach a state that looked even remotely like normal and it is probably permanently hypoplastic. Functional tests of the lymphatic tissue, moreover, in our hands give similar indications: the capacity of the animal to reject homografts of tumour (Barnes *et al.*, 1957; Ilbery, Koller and Loutit, 1958) and of skin (Bridges, Loutit and Micklem, 1960) is lost for some two months or more.

[illegible]

ferent lymphocytes. The experimental data of Hamilton (1959) and others on the rate of loss of labelled lymphocytes are comparable with there being two populations with different half-lives.

The radiation chimeras formed of cell populations of two genetically different origins may, like Billingham and Brent's chimeras, surmount the immunological disturbances initiated by the graft-versus-host reaction. This is particularly the case when host and graft cells though genetically different have identical H-2 T antigens. The majority of our CBA/T6+ inter-strain chimeras live for a substantial number of months up to nearly two years. This is probably because, whilst the T6 is selected only on the basis of its translocated chromosome, the + element is selected for H-2^k, the same antigen carried in homozygous combination by CBA. Our experience is that these animals originally irradiated at about 100 days old with 950–1,000 rads retain the marked donor cells as their haemopoietic populations in bone marrow, spleen and lymphoid tissue (in so far as mitoses are detected at all in the hypoplastic lymph tissue) for the rest of their lives. It seems therefore that this dose of X-rays in this variety of mouse is sufficient permanently to inactivate the host's immunologically functioning tissue. The same may well be true of the heterologous chimera, mouse/rat, of our laboratory, but so few of them survive "secondary disease" that it is difficult to be dogmatic (Barnes *et al.*, 1959*a*). However, we observe that if rather smaller doses of X-rays are given before restoration with rat's bone marrow, although the initial recolonization is much the same, the late results are often different. After 850 rads, for instance, which is about the LD_{60}, within two to three months the marrow and lymphatic tissues may have reverted partially or wholly to murine tissue.

Our thesis is that the partial reversion is indicative of recovery

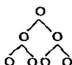
the present writer has of the histology is of more than usually active granulopoiesis with adequate erythropoiesis and thrombopoiesis. Examination of further sections plus smears will be necessary. The peripheral blood is certainly deficient in circulating lymphocytes.

The graft-versus-host reaction is not confined to the radiation chimera. Billingham and Brent (1959) have identified it as the cause of "runt disease" when foreign spleen cells are injected intravenously into newborn mice. The hosts become immunologically tolerant of the grafted cells, which being lymphoid in nature are capable of immunological reaction against the host. The clinical and histological picture of "runt disease" is remarkably similar to that of "secondary disease of the radiation chimera". However, Billingham and Brent find that by substituting bone marrow for spleen as the cells for induction of immunological tolerance in the host they avoid runt disease or reduce it so markedly that it is no longer a clinical obstacle in their experimentation. From this angle therefore the lymphoid tissue of bone marrow, like that of thymus, seems less susceptible to activation by T antigens than splenic lymph tissue.

Our further rather different experience (Bridges, Loutit and Micklem, 1960) is similar. Isologous CBA radiation chimeras will sustain a skin graft of homologous murine skin, e.g. of strain C57BL, for some two months, but if the irradiated CBA mouse is restored with bone marrow from another CBA mouse hyperimmunized by injections of C57BL cells, the resultant isologous chimeras will retain C57BL skin for some six weeks only or for considerably less if the restoration has been made with bone marrow and spleen from a hyperimmune animal. Our conclu- [illegible] in transplanta- [illegible] f its [illegible], the lymphocytic elements which are immunologically active, this would make marrow comparable, as noted above, with thymus,

been killed or undergone their natural development, as for example by equal maturation of both daughter cells at division.

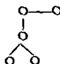
Model 1



Stem cell
Maturation stage 1
Maturation stage 2

The alternative must be some regenerative process, either by an asymmetric division, e.g.

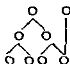
Model 2



Stem cell
Maturation stage 1
Maturation stage 2

or by recycling with dedifferentiation, e.g.

Model 3



Stem cell
Maturation stage 1
Maturation stage 2

Another approach through radiation chimeras has also led us to regard Model 1 with disfavour. Preliminary communication of this approach has already been made (Barnes, Ford and Loutit, 1959). A CBA mouse was irradiated with 950 rads of X-rays in February 1956 and restored by injection of some 10^6 nucleated cells from spleen of an infant T6+ mouse. A year later it was sacrificed and about 10^6 nucleated cells from its bone marrow (now T6+ cells according to cytological evidence) were used to restore a second irradiated CBA mouse. The process has been repeated at annual intervals till now, when the fifth generation of passage is in train. For each 10^6 cells of inoculum there can on classical teaching be but a small proportion of stem cells. Nevertheless our procedure has resulted in each generation in a number of suitably long-lived and therefore presumably haematologically adequate chimeras.

of the host's haemopoietic and lymphopoietic tissue arising from damaged, but not irrevocably damaged, stem cells. These cells in spite of the damage received must be physiologically at least as competent in their native environment as the normal donor cells in, to them, a foreign environment. Since they do not entirely eliminate the donor population they must be immunologically unreactive and perhaps specifically tolerant to them.

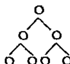
On the other hand when complete reversion occurs it is probably a manifestation not only of cellular recovery but also of functional immunological recovery. The host then stages an indolent reaction against the graft, eliminating it slowly enough to permit survival of the animal in spite of the change of effective haemopoietic populations.

In some instances of such reversion the damage that the stem cells have suffered from the radiation may be illustrated by the new population of host's cells which have visible chromosome translocations (Barnes *et al.*, 1959*b*). Each of these translocations is specific and clones of cells all having the same lesion are to be found. These clones must each have arisen from a clone-mother cell which sustained that particular non-lethal lesion. It is notable that mitotic cells having the same signature are to be found in bone marrow, spleen, lymph node and thymus. The clone-mother cell must, therefore, have been sufficiently primitive to have multi-, perhaps toti-, potentiality for haemopoiesis. It would appear that the reverted haemopoietic tissues must have developed ultimately from some two, three or a few more stem cells.

This conclusion that virtually all haemopoietic tissue, even though in some categories hypoplastic, could be attributed to the products of a mere handful of stem cells would seem to rule out any hypothesis that haemopoietic stem cells in the free-living animals are limited in number, as, for instance, the number of ova are thought to be finite. Senility of the ovary is a *fait accompli* when all ova are discharged. It was conceivable that haemopoiesis could similarly grind to a stop when all available stem cells had

been killed or undergone their natural development, as for example by equal maturation of both daughter cells at division.

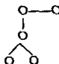
Model 1



Stem cell
Maturation stage 1
Maturation stage 2

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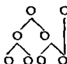
Model 2



Stem cell
Maturation stage 1
Maturation stage 2

or by recycling with dedifferentiation, e.g.

Model 3



Stem cell
Maturation stage 1
Maturation stage 2

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DISCUSSION

Lajtha: I should like to raise a basic principle: what happens at cell division? Dr. Loutit's model No 2 implies that there is a stem cell which divides, and that one of the daughter cells will differentiate and the other remain a stem cell This is a mental picture which I find [illegible]

[illegible]

into only one of the daughter cells. Unless there is very good experimental evidence, I do not like the idea. I would like to offer a concept which would have the same statistical result, without involving the mother cell in this curious responsibility of developing something asymmetrical. This concept is that the stem cell is an innocent population minding its own business—the only thing it does know is to divide [illegible]

[illegible]

formation in many other conditions So from this stem population any one cell, whether before or after division, can be persuaded by a specific stimulus (of "differentiation") to produce a specific enzyme system and go in the erythroid, myeloid or thrombocytic direction.

Yoffey. Must the stem cells be in the same situation as the other differentiating cells, or can they be elsewhere and migrate through the blood? Your idea is basically the same as that of Maximow, with the exception that he made his separate stem cell population migrate through the blood stream.

Lajtha. That can be tested experimentally. The fact that leukaemoid peripheral blood (or perhaps even normal peripheral blood) can

apparently recolonize marrow means that probably it can go through the peripheral blood. What this cell is in the peripheral blood is a different question.

Yoffey: It is R. M. Merwin's paper (1959. *Proc. Soc. exp. Biol. (N.Y.)*, **101**, 9) that you have in mind.

Cronkite: I think the experiments of M. N. Swift, S. T. Tabeta and V. P. Bond (1954. *Radiat. Res.*, **1**, 241), in which the upper half of the animal was irradiated while the lower was shielded, and immediately afterwards the lower was irradiated while the upper half was shielded, are conclusive proof that something has circulated in a very short period of time and found refuge under the lead shield. Enough of ... I see no ... must cir- ...

We had the opportunity to observe the bone ... the five human beings who were sublethally irradiated, and so far as we could ascertain there was no influx of cells which could be described as small ... the time of recovery.

... thymus, lymph node, Peyer's patches, and ... chamber of the eye in animals irradiated to a degree that will eradicate secondary immune response. He has found that these animals have their ability to produce antibodies restored almost completely by these tissues, though it is less well, or perhaps not at all, restored by the bone marrow. On the other hand, it is only the bone marrow or the spleen with myelopoiesis that results in an increased survival rate following irradiation.

Yoffey: R. D. Stoner and W. M. Hale (1955. *J. Immunol.*, **75**, 203) concluded that the reason why the thymus did not normally produce ... simply that somehow antigens did not get to it, but if ... then it would ... Dr. Loutit was making

Cronkite. Stoner has always worked with a soluble antigen that easily gets to these transplants. These experiments have not been repeated with particulate antigens that are phagocytized in the reticulo-

endothelial system and do not have an equal opportunity of getting to the intraocular transplant.

Everett: To my knowledge the only report of a successful transplant of lymphopoietic tissue providing protection following lethal irradiation is that of Dr. E. Salvidio of Italy who administered calf thymus to rats. In his opinion it is the reticulum cell that repopulates the bone marrow.

Loutit: I agree with you, Dr. Lajtha, that cells can redistribute themselves and metastasize and so on, but would you support me in ruling out model No. 1? We have four generations of mice, four mouse-years of life, all supported by an original inoculum of about one million cells, of which perhaps 50 per cent were small lymphocytes because the original inoculum came from an infant spleen, and the rest were the usual mixed bag of cells some of which you can name and some of which you cannot. That means a total of about a million cells which could be stem cells by one means or another—differentiation, dedifferentiation [illegible]

to [illegible]

ably depleted when you irradiate. When you inoculate a stem cell population, whether it is a million or even, theoretically, one stem cell, the cells will keep dividing in a purely geometric fashion, remaining stem cells. Eventually they will fill up the space. Of course there is the stimulus for differentiation—erythropoietin, myelopoietin, etc.—which will pick on the odd cell (depending on the concentration of the material in the plasma) and determine what proportion of the population present will be affected The affected cells will be pulled out of the stem compartment because they start differentiating—they may undergo two, three or four divisions while they differentiate, but they cease to be stem cells. So you can delay the repopulation of the stem compartment by fishing cells out from it for differentiation, but this will not prevent it unless you repeatedly remove more than 50 per cent, in which case of course, the stem population will be depleted.

Leblond: Statistically, this is actually the same as you are doing.

Lajtha: Yes.

Stohlman: But it is not the same physiologically. Also, if you give doses of radiation within the lethal range the delay in recovery in the

anaemic animal is such that it would suggest that maybe you have to fill up this space before any differentiation occurs, even though erythropoietin, or what have you, is there, then all of a sudden repopulation with myeloid and erythroid elements returns with a surge.

Lajtha: That is quite possible. Furthermore if you keep repeating the radiation before the stem compartment has filled up you can delay the recovery, until in fact there is no recovery at all.

Lamerton: Thus, formally, is a nice theory. But what physical picture have you of a stem cell compartment that has to be filled?

Yoffey: The stem cell compartment is, for example, the lymphoid tissue, according to Maximow. Under normal conditions, erythropoietic and granulopoietic stimuli do not get to it. But now and again they do, and then they start differentiating *in situ*, undergoing erythroid or myeloid metaplasia. Identifying this mysterious stem cell is a fascinating problem. We all talk about it and it is very hard to pinpoint. I combed the transfusion literature. I thought that if people were giving marrow transfusions surely they must have published analyses, so that one could look through them and see what were the possible stem cells that might be responsible. The only detailed analysis of a marrow transfusion which I could find was in a fairly recent paper by E. L. Alpen and J Baum (1958. *Blood*, 12, 1168). They transfused autologous marrow into lethally irradiated dogs. In the process of transfusing the dog with its own marrow for protection they also made differential and total counts. They commented that most of the cells which they gave were post-mitotic and not much good from the point of view of what they call regeneration potential. They had: 0·9 per cent myeloblasts, 7 per cent lymphocytes, 1·3 per cent erythroblasts, 1·1 per cent unclassified, and 0·2 per cent reticuloendothelial cells. The reticulum cell does not figure there and they did not take any account of damaged cells.

Are there any other detailed analyses of marrow transfusates in the literature?

Jacobson. We looked at the spleen, and I decided that it was impossible to classify the cells. The same was true of the embryonic blood-forming tissue, so it seemed ridiculous to put down numbers when I was not sure what was what, and I simply left it as so many cells. I was under the impression that E. Lorenz did some work on the various

constituents of the bone marrow, but I don't remember having seen the publication.

Yoffey: But in one of your own papers (Jacobson, L. O., Marks, E. K., and Gaston, E. O. [1955]. *In* Radiobiology Symposium, 1954, p. 122, ed. Bacq. Z. M. and Alexander, P. London: Butterworth) you made an interesting comment, and gave a known number of cells. I think you said the cells from younger animals—the transfusates—were something like three times as effective in giving protection as those from older animals. I do not know the details but the striking difference that one usually finds in the younger animals is the increased numbers of lymphoid cells. I am merely voicing my own inability to get enough data on which to begin to know where to look for the protecting cells. Having these figures of Alpen and Baum, we then, using our quantitative data, tried to calculate what degree of marrow repopulation we would get in the dog. It was ridiculously low—something like 1,000 lymphocytes/mm.^{3} of marrow, and about 100 myeloblasts/mm.^{3}, but at least if the marrow lymphocytes could repopulate the lymphoid tissues fairly quickly, and fill up the stem cell compartment, 1,000 lymphocytes might in fact be worth a good deal more than they would otherwise be I advance this simply as evidence of the sort of difficulty one has in trying to analyse the situation.

Stohlman: There have been a couple of recent observations which might bear on the "filling up" of a stem cell pool. N. T. Shahidi and L. K. Diamond (1959. *A.M A. J. Dis. Child.*, 34, 293) have treated aplastic anaemia, in children, with testosterone and corticosteroids They have to give it for two or three months before they observe a response and then all of a sudden there will be a surge of cells out, much as with irradiation only the delay is longer With a hypertransfusion on the other hand, if you give a stimulus, the cells come out within two or three days. It would seem that with steroid therapy and irradiation one must first fill up a void and then differentiation occurs.

Yoffey: Did Diamond finally identify his stem cell?

Stohlman: Not to my knowledge. The lymphoid tissue was normal in these children

Cronkite: Dr. Fliedner now has some work in progress in which he is using the transfusion of *in vitro* thymidine-labelled bone marrow cells in rats. The types of cells labelled (myeloblasts, promyelocytes,

erythroblasts, etc.) should divide once again. He has transfused this labelled bone marrow (*incubated in vitro* for one hour and washed with thymidine-free plasma) into otherwise fatally irradiated rats, and killed the recipients at regular intervals. The recipients' marrow has been studied for the presence of labelled cells. Thirty-three hours after transfusion he has found labelled myelocytes and metamyelocytes with a grain count on the average about half that of the original labelled cells, indicating that the transfused cells had divided once and undergone further differentiation. To date he has not identified the stem cell. However, by this approach, applying quantitative cytological methods, long advocated by Prof. Yoffey, I feel there is some chance of identifying the elusive stem cell.

I am really confused at what Dr. Loutit and Dr. Lajtha have said. We have talked about a possible primitive proliferating pool of cells which migrate through the body via blood and lymph, and which we believe can respond to a peripheral signal and mature and multiply in almost any direction. We have absolutely no direct evidence for it—it is just an idea, comparable to the ideas haematologists have had for [illegible] single stem [illegible]

Loutit: I am certainly postulating that within the body there is a totipotent haemopoietic cell, which given the right stimulus can ultimately produce in the marrow either red cells or granulocytes or platelets, and, in lymphoid tissue, lymphocytes. But what that cell looks like, and how to recognize it, I just do not know. We can identify the later product by means of its chromosomes; but what it originally looked like as a histological or cytological preparation I do not know.

Everett: With respect to Dr. Lajtha's comments and his concern over the potential of this mother cell, it would appear to me that there are other areas outside the haemopoietic tissues in which this type of thing does occur, in the germ cell line for example. If one carried this concern to the ultimate I fail to see that we could ever get any embryonic differentiation without this potential.

Lajtha: It rather depends which cell is hit by the respective molecule.

Everett: You were concerned about the ability of one cell to give rise to two different types, as in Dr. Loutit's model No. 2.

Lajtha: In the intestinal epithelium, for example, you may have an exponentially growing group of cells (crypt cells). Now, if by chance any crypt cell is pushed to a certain height in the villus, either away from a capillary or within a certain distance in the lumen, it may have a higher chance of being hit by a particular molecule, or simply of arriving in a milieu in which it cannot divide any more. We have in fact statistically the same event that model No. 2 would depict, without implying that one cell has to divide asymmetrically—it just means you push that cell into a surrounding where it can meet the necessary stimulus or milieu for differentiation. Once it meets it, it is a different cell, but not because it divided into one or the other.

The other point is that basophilic normoblasts in the marrow, which are highly dividing cells with 60, 70 or 80 per cent DNA synthesis, cannot be self-maintaining populations, unless Dr. Loutit's model No. 3 works, and after a certain number of divisions they dedifferentiate. This is because every single cell of this population synthesizes haemoglobin. You can measure the iron uptake and the haemoglobin content increases. But if they keep on dividing and synthesizing haemoglobin, they cannot remain the same basophilic normoblasts synthesizing haemoglobin—they are bound to become polychromatic at one stage or another. That is, unless something happens, and they suddenly lose all their haem content and go back to haemocytoblast, pronormoblast or whatever cell you like to call it. They could not be a self-maintaining population without dedifferentiation—and, of course, there is no good evidence for dedifferentiation *in vivo*.

MODELS FOR LYMPHOCYTE AND PLASMOCYTE FORMATION

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Maximow (1909) and a majority of authors consider the cells of the thymus to be genuine lymphocytes. Indeed, the various types of "lymphocytes" found in the thymus have the same appearance as those found in lymph nodes and other tissues. In these various locations, they can be classified into three types, according to the classification introduced by Maximow (1909): large lymphocytes or lymphoblasts, medium lymphocytes or prolymphocytes, and small, so-called mature lymphocytes. It seems that the large lymphocytes present in tissues seldom appear in the blood stream, but medium and small lymphocytes are normally found in the circulation, where they are referred to as large and small lymphocytes, respectively, by clinical haematologists.

Little is known of the mode of formation of lymphocytes in the adult thymus. Current opinion is that the smaller the lymphocyte the more mature it is. Large lymphocytes would give rise to medium ones, which in turn would give rise to small lymphocytes Dustin (1913) investigated the embryology of the amphibian thymus and observed that the first lymphocytes to appear in the course of development were large lymphocytes. With the passing of time, smaller and smaller lymphocytes were seen to arise from mitosis. Dustin felt that a variable number of generations, perhaps five to eight, would be required for a large lymphocyte to evolve into small lymphocytes. On the other hand,

Maximow believed that medium lymphocytes were the main source of small lymphocytes, while large lymphocytes would play a negligible rôle in this regard (1932).

As for the reticular cells, their participation in lymphocytopoiesis is likely but requires confirmation. According to Dustin (1913), the embryonic thymus contains reticular cells which are initially epithelial and eventually produce large lymphocytes. Baillif (1949) described the production of lymphocytes from "epithelial" reticular cells during regeneration of involuted thymuses Maximow (1932) as well as Downey (1948) and recently Kindred (1955) and Trowell (1957) expressed the belief that at least some reticular cells occasionally yield lymphocytes in the adult.

Much of the classical work on lymphocytopoiesis consisted of patient cytological observations dealing with normal and pathological lymphocytes. The slow progress in this field may indicate a need for new approaches. Indeed, quantitative results of Andreasen and Ottesen (1945), Kindred (1955), and Osogoe, Monden and Ito (1957) revealed the existence of numerous mitoses of thymic cells; and these authors concluded that the thymus is a more active producer of lymphocytes than lymph nodes or spleen. Nevertheless, many haematologists still believe that thymic lymphocytes are not released into the circulation, perhaps because it is not known how lymphocytes leave the thymus, if they do so at all (Policard, 1950). Hence the often-expressed thought that the fate of thymic lymphocytes is to degenerate locally, and, thus, to release deoxyribonucleic acid into the circulation (Dustin, 1913, 1920), although the considerable amount of work done in support of this hypothesis (Kelsall and Crabb, 1958) has only provided circumstantial, unconvincing evidence.

In the present work the approach used has been to count resting and dividing cells of the various types. Then a model describing lymphocyte formation in terms fitting the results of the counts as closely as possible was designed. In addition, observations were

made which indicated how thymic lymphocytes may pass into the circulation.

Towards the end of the present article, brief mention will be made of the application of the same method to the plasma cells—or plasmocytes—found in the thoracic lymph nodes of the rat. A model for the formation of these cells will also be presented

Technique

The thymuses of 28 normal, 10-week-old, male albino rats were fixed in Bouin-Hollande; 5 μ-thick sections were prepared and stained with Dominici. Using these sections, the volumes of the two main thymic regions, cortex and medulla, were measured and found to account for 75 per cent and 25 per cent of the thymic parenchyma, respectively. The data obtained in cortex (Sainte-Marie and Leblond, 1958*a*) and medulla (Sainte-Marie and Leblond, 1958*b*) will be presented in turn.

The same procedure was applied in a study of plasmocytes in the medullary cords of thoracic lymph nodes. These nodes were obtained from 32 normal, 10-week-old, male albino rats. They were fixed and stained in the same manner as the thymuses.

Cortex of thymus

The cells seen in the cortex were classified into four main types: reticular cells, and large (lymphoblasts), medium (prolymphocytes) and small lymphocytes (Fig. 1). The reticular cell had a large and pale nucleus with a usually colourless and not clearly delimited cytoplasm. The large lymphocyte showed a less pale nucleus, exceeding 5·9 μ in diameter, which was surrounded by a relatively abundant, well-limited and basophilic cytoplasm. The still darker nucleus of the medium lymphocyte varied between 4·5 and 5·9 μ, and was surrounded by a well-delineated, but less abundant and less basophilic cytoplasm than in large lymphocytes Finally, the very dark nucleus of the small lymphocyte reached

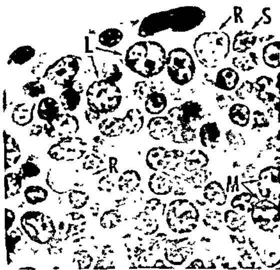


FIG. 1 Cortex of rat thymus Note the nuclei of reticular cells (R), which are pale and show no distinct cytoplasm, large lymphocytes (L) with basophilic cytoplasm, medium (M) and small lymphocytes (S) Small lymphocytes are plentiful

up to 4·5 μ, while its scanty cytoplasm was hardly seen. Resting [illegible] counted.

[illegible] the various regions of the cortex (total cortex; Table I) showed that reticular cells were rare and the number of large, medium and small lymphocytes increased in this order.

Table I

RELATIVE NUMBER OF CELLS, MITOTIC INDEX AND CALCULATED LIFESPAN OF EACH CELL TYPE IN "TOTAL" CORTEX OF RAT THYMUS

	Relative No. of cells (per reticular cell)	*Mitotic index (%)*	*Lifespan* (hours)*
Reticular cell	1·0	1·5	66·7
Large lymphocyte	3·3	6·5	15·8
Medium lymphocyte	5·9	13·1	7·6
Small lymphocyte	81·3	1·5	66·7

* The lifespan is obtained from the mitotic index on the assumption of a one-hour mitotic duration.

The *mitotic index*, that is, the ratio of the number of dividing to the number of resting and dividing cells, was calculated for each cell type in the cortex (Table I). Each cell type proved to have a relatively high mitotic index (1·5 to 13·1 *per cent*). Such a high mitotic index might be due to growth, as in the case of hair follicles, but then growth is extremely rapid as shown by elongation of the hair. No such increase in size takes place in [illegible]

rate but mitoses produce new cells which balance the loss. On the contrary, cell populations which are not being renewed, like the parenchymal cells of the liver, show only rare mitoses providing for a slow growth of the organ. Thus, the mitotic index of liver parenchymal cells is of the order of 0·1 per cent. It was therefore concluded from the high mitotic indices found in the four cell types of the thymic cortex that each one of them is being

renewed. This conclusion implied that the cells being formed by mitosis in the cortex did not accumulate there, but were eliminated in one way or another. Let us see how this idea fits in with, for instance, the data on the reticular cells.

Assuming that the reticular cells of the thymic cortex are a homogeneous population, their mitotic index (1·5 per cent) indicates the frequency with which each cell divides. From this figure, the chance for a reticular cell to be seen in mitosis may be calculated to be 1 in 66·7. If we were to assume further that one hour is the time required for completion of a mitosis, 66·7 hours would be the duration of the lifespan of the cell (the lifespan of a renewing cell, also referred to as "generation time", is defined as the time elapsing between the end of the division of its mother cell and the end of its own division). *If each reticular cell were to last 66·7 hours, that is, nearly three days, then, on the average, the stock of reticular cells would be duplicated every third day.* However approximate this figure may be, it remained clear that reticular cells divided at a rapid rate. Obviously, if the new cells were to be retained in the tissue, they would soon fill up the organ. Some of the cells must therefore be eliminated. This may be done in three ways, reticular cells could either degenerate, leave the cortex, or transform into another cell type. No sign of degeneration or migration of these cells was seen in the cortex, and therefore it was concluded that they must transform into other cells. The existence of transitional forms between reticular cells and large lymphocytes led to the conclusion that the former transform into the latter. (The reverse possibility—large lymphocytes transforming into reticular cells—was unlikely, since the short-lived large lymphocytes would then tend to be less rather than more numerous than reticular cells: Table I)

Similar reasoning was applied to the other thymic cells. It was thus concluded that large lymphocytes were rapidly renewed (lifespan estimated to be 15·87 hours for a one-hour mitotic duration) and gave rise to medium lymphocytes. These in turn

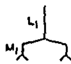
must be renewed at an even faster rate (lifespan 7·63 hours for a one-hour mitotic duration) and give rise to small lymphocytes. But what happened to small lymphocytes? They neither transformed into other cells, nor did they degenerate in the healthy thymus. The only alternative was that they found their way out of the thymic cortex. This conclusion will be examined below.

The next step was to determine the *number of generations* through which the cells of a given type have to go before transforming into the next cell type. This was done by considering the number of dividing cells of each type (Table II). Several assumptions

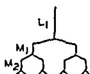
Table II
RELATIVE NUMBER OF MITOSES OF EACH CELL TYPE IN "TOTAL" CORTEX OF RAT THYMUS

Reticular cell	1
Large lymphocyte	14
Medium lymphocyte	53
Small lymphocyte	71

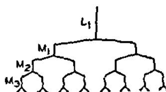
were made, namely, that the lifespan was the same for all cells of a given type, that mitotic duration was the same for all cell types, and, finally, that only small lymphocytes left the thymic cortex in substantial numbers (Sainte-Marie and Leblond, 1958*a*). Under these conditions, precise conclusions regarding the number of generations may be reached. For instance, let us consider the simplest possibility—a single generation of large (L_1) followed by a single generation of medium (M_1) lymphocytes, as represented in diagram I of Fig 2. Any large lymphocyte would then divide to give rise to two medium lymphocytes, which would in turn divide to produce four small lymphocytes. Under these conditions sections should show on the average twice as many mitoses of medium as of large lymphocytes. This is not in agreement with the experimental data which show 3·8 times as many mitoses in medium as in large lymphocytes. Many other possibilities were considered. For instance, for diagrams II, III, IV, V and VI, the ratios of mitoses of medium over those of large



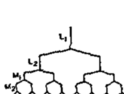
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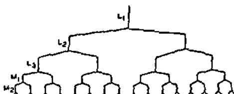
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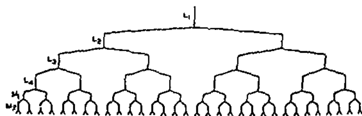
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IV



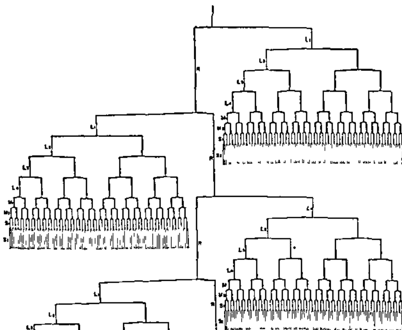
V



VI

are indicated on the diagrams by the junction of a vertical and two oblique lines (Vertical dimension in this and following diagram indicates time)

lymphocytes were 6, 14, 4, 3·4 and 3·2. Only the last three figures came close to the experimental data. Hence, there were



two generations of medium for two, three or four generations of large lymphocytes.

Similar analyses of all the figures available made it possible to narrow down the number of possibilities. Finally, it was concluded that the relative numbers of generations of large, medium

and small lymphocytes were four, two and two respectively, while reticular cells behaved as indicated in the model presented in Fig. 3 (Sainte-Marie and Leblond, 1958*a*).

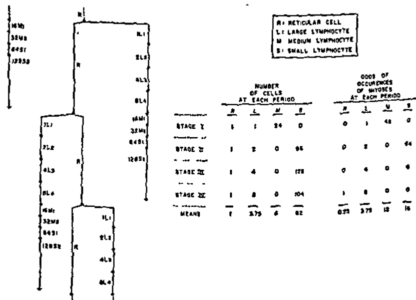


FIG. 4. Simplified representation of the lymphocyte-formation model of Fig. 3. Each series of generations arising from the division of a reticular cell ends with an arrow, which is taken to indicate the mean time when small lymphocytes leave the tissue

The number of cells and the odds of occurrence of mitosis were estimated at four periods—referred to as stages This was done by inspection of the corresponding portion of the diagram The means obtained for each cell type were used to calculate the "expected" ratios in Fig. 5.

According to this pattern, on the average each reticular cell divides to produce a large lymphocyte and another reticular cell. Before the new reticular cell enters, in its turn, into division, the

EXPECTED
EXPERIMENTAL
LYMPHOCYTE FORMATION IN THYMUS

CELLS MITOSES

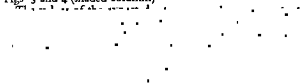
RATIOS

LARGE / RETICULAR

MEDIUM / LARGE

SMALL / MEDIUM

FIG 5 Comparison between the experimental ratios obtained for the number of lymphocytes and their mitoses in thymic cortex (dark columns) and those expected from the model in Figs 3 and 4 (shaded columns)



model)

large lymphocyte undergoes a series of divisions yielding four generations of large lymphocytes. Then, each large lymphocyte of the fourth generation gives rise to medium lymphocytes of which there are two shorter-lived generations which yield a first generation of small lymphocytes. These divide once, to yield a total of 128 mature non-dividable small lymphocytes, which must eventually leave the cortex. This whole cycle is repeated indefinitely. The means of the numbers of cells and mitoses which should be found if this pattern is correct are presented at the lower right of Fig. 4 (which depicts the pattern in a simplified form). The ratios of these means—referred to as "expected" values—are compared to the "experimental" data in a series of diagrams (Fig. 5). Clearly, expected and experimental data are in fair agreement, since the expected ratios were within the standard errors of the experimental ones (except for the numbers of mitoses of medium over large lymphocytes, which were on the borderline). Therefore, the proposed model for lymphocyte formation in the thymus cortex receives support from this statistical analysis

Medulla of thymus

The medulla showed the four cell types seen in the cortex, but with a few minor differences in appearance. The nuclei of some reticular cells contained granules of chromatin-like material, often present within vacuoles. Jolly (1923), who first described such nuclei, interpreted them as undergoing degeneration. Indeed other stages of degeneration up to complete lysis of the nuclei were commonly seen, either in isolated cells, or in reticular cells making up the structures known as Hassall's corpuscles.

The large and medium lymphocytes, except for a few irregularly shaped nuclei, were identical to those seen in the cortex. Irregularities of nuclear shape were pronounced in small lymphocytes. Some nuclei of small lymphocytes sent off several processes and even appeared crenated (Fig. 6, A). Other nuclei

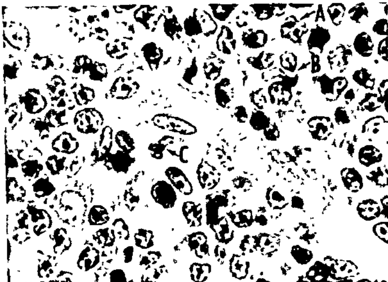


FIG. 6 Medulla of the rat thymus. Note the irregular appearance of many nuclei The nuclei of small lymphocytes may have a crenated appearance (A) or send off long (B) or short (C) processes.



FIG 7 [illegible] branes [illegible] of "per [illegible] appeara [illegible] vessel (BV) is almost completely surrounded by that of the lymphatic channel (LV) Scattered reticular fibres and membranes may also be seen in the tissue

facing page 162

Fig 8 Medulla of rat thymus, with a blood vessel at lower centre. The arrow points to a small lymphocyte located in a perivascular lymphatic channel and sending off a process across the wall of the blood vessel

showed a single, large, pseudopod-like process (Fig. 6, C). In Fig 6, the nucleus of a small lymphocyte is seen to send off a long, squirrel-tail-like process (B). These irregular forms, which incidentally were rarely seen in the cortex, were interpreted as indicating pronounced amoeboid activity of the cells in the medulla.

It is generally claimed that the thymic parenchyma contains only a few or no lymph vessels (Jolly, 1923; Policard, 1950). Indeed, typical lymph vessels were seldom seen, but numerous lymphatic spaces were found to be associated with blood vessels in the medulla and in the trabeculae. In cross-section, such spaces were seen to surround blood vessels, which were thus enclosed as in a coaxial tube (Fig. 7) Hence, they were called "perivascular lymphatic channels". These channels were enclosed by thin, barely visible, endothelium-lined walls, within which rows of lymphocytes were seen. An interesting observation was that the walls of these channels often showed cells undergoing diapedesis, and so did the walls of the blood vessels. Most diapedetic cells were small lymphocytes (Figs. 8 and 9). Occasionally larger lymphocytes were seen, but never reticular cells.

One of the illustrations shows a small lymphocyte outside a blood vessel, with a nuclear process across the vessel wall (Fig. 8) and another one within a blood vessel, again with a nuclear process in the wall (Fig. 9). These figures are interpreted as stages in the passage of small lymphocytes to the blood stream It should be emphasized that such pictures of diapedesis were not seen in the cortex. Therefore, it is possible that the medulla contains a factor, possibly secreted by the dying reticular cells, that is responsible for the pronounced amoeboidism and diapedesis observed in this region.

To find out the pattern for the mode of lymphocyte formation in the medulla (Sainte-Marie and Leblond, 1958*b*), the ratios of cells and mitotic counts were analysed by the same approach as was used in the cortex. There were several important similarities

between cortex and medulla. Thus, except for small lymphocytes, the mitotic indices were comparable in both regions (0·9, 5·3, 14·3 and 0·7 per cent respectively for reticular cells, large, medium and small lymphocytes in the medulla). This observation indicated that reticular cells, as well as large and medium lymphocytes, had a comparable lifespan in cortex and medulla and also that all these cells were continuously renewed in the medulla as in the cortex. Therefore, it was felt likely that lymphocyte formation evolved in a similar way in both regions. To examine this hypothesis, the number of cells of each type was compared to that of its precursor in the medulla. Whenever the relation differed from that found in the cortex, an explanation was sought in the cytological and architectural features characteristic of the medulla.

Table III gives the comparison between the number of cells found in the medulla and those expected if lymphocyte formation

Table III

NUMBER OF CELLS IN THE MEDULLA OF THE RAT THYMUS, AS COMPARED WITH THE NUMBERS EXPECTED FROM THE POPULATION OF THE IMMEDIATE PRECURSOR

	Cell counts	*Expected*	*Difference*
Reticular cells	0·790	0·790	—
Large lymphocytes	0·025	2·610	−2·560
Medium lymphocytes	0·114	0·045	+0·069
Small lymphocytes	9·040	0·840	+8·200

were to proceed according to the pattern devised for the cortex. Thus, the large lymphocytes were expected to be three times as numerous as the reticular cells, but were in fact much less abundant, only 1 per cent of the expected number. It was therefore concluded that the reticular cells which are continuously being produced by mitosis did not usually transform into large lymphocytes. Since they did not leave either, the only possibility was that they degenerated and died; and indeed degenerating forms of these cells were often found. Some died isolated, others in

Hassall's corpuscles (these corpuscles may be considered to be dynamic structures into which some of the reticular cells aggregate and eventually degenerate).

The number of medium lymphocytes was about twice and the number of small lymphocytes about ten times as large as expected (Table III). It was concluded that half of the medium, and nine in ten of the small lymphocytes found in the medulla came from somewhere else. Where could they come from? Observations of Regaud and Crémieu (1912) on the irradiated thymus of the cat led these authors to postulate that, under physiological conditions, lymphocytes migrate from cortex to medulla. Jolly (1923) and Policard (1950) also shared this opinion. A strong argument in favour of this theory was mentioned above, that is, the small lymphocytes formed in cortex have to leave this zone. But, since no diapedesis was seen to occur in the cortex, they must leave this zone in some other way; and the only possibility seems to be for them to migrate to the medulla.

On reaching the medulla, lymphocytes would become endowed with the capacity for active amoeboid motion and would then leave by entering the numerous lymphatic channels and blood vessels of this zone. From the figures quoted in the previous paragraph, it was concluded that about 90 per cent of the small and 50 per cent of the medium lymphocytes present in the medulla came from the cortex. Thus, few lymphocytes would be formed in the medulla, which appeared to be mainly a passage-way for these cells to reach the circulation. This concept is consistent with the observations of amoeboid activity, and of diapedesis across the walls of the perivascular lymphatic channels and blood vessels in the medulla.

Our conclusions regarding the respective rôles of the cortex and medulla in lymphocyte formation in the rat thymus are illustrated by the diagram in Fig. 10. The left part refers to the cortex, and the right to the medulla. The base of the diagram is curved to the right to indicate that mature small lymphocytes arising in the

FIG 10 Pattern for the formation of lymphocytes in the rat thymus, and for the migration of mature small lymphocytes into the medulla (lettering as in Figs. 2 and 4)

The basic pattern ...

other half divide are ...

blood and lymphatic circulation The diagram assumes that only S_2 cells migrate although some degree of migration of large, medium and small (S_1) lymphocytes to the outside also occurs, but apparently to a lesser extent.

cortex migrate towards and into the medulla, where they join with those formed in this region. That the line representing the cell filiation in the cortex is much thicker than that for the medulla indicates that many more thymic lymphocytes are formed in the cortex than in the medulla. The pattern for the medulla is changed as far as reticular cells are concerned, since their mitoses usually yield new reticular cells, which eventually divide again, or degenerate. The various types of lymphocytes would reproduce in the same manner as in the cortex. The few small lymphocytes formed in the medulla join the numerous ones arising from the cortex to enter the circulation by diapedesis through the walls of blood vessels and lymph channels.

Conclusions for the thymus

Resting and dividing cells were counted in the cortex and medulla of thymus of 10-week-old male rats. The cells were of the same four types in both regions. Moreover, except for the small lymphocytes, each cell type had nearly the same mitotic index, and therefore the same lifespan, in both regions. The data indicated that, throughout the thymus, the small lymphocyte [illegible]

[illegible]

number of large lymphocytes present, it appeared that the evolution of the reticular cells into lymphocytes was the exception, instead of being the rule, as in the cortex. The overproduction of reticular cells was found to be balanced by the degeneration of single and Hassall-grouped reticular cells.

The presence, in the medulla, of *many more small lymphocytes* than expected from the number of medium ones present, was taken to indicate that small lymphocytes migrate from the cortex into this zone. Diapedesis of small lymphocytes was commonly seen across the walls of "perivascular lymphatic channels" and of

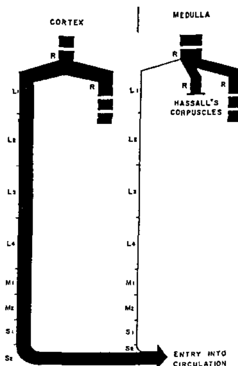


FIG 10. Pattern for the formation of lymphocytes in the rat thymus, and for the migration of mature small lymphocytes into the medulla (lettering as in Figs 2 and 4).

The basic pattern pictured in Fig 4 is repeated for cortex and medulla, with the thickness of the long vertical portion made proportional to the amount of local lymphocyte production At the top of the diagram, reticular cells are shown dividing In the cortex each such division yields, on the average, a large

blood and lymphatic circulation The diagram assumes that only S_2 cells migrate although some degree of migration of large, medium and small (S_1) lymphocytes to the outside also occurs, but apparently to a lesser extent

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the blood vessels in the medulla, but not in the cortex. Cortex-formed small lymphocytes would have to migrate to the medulla before entering the circulation. Thus, while a small degree of lymphocyte formation occurs in the medulla, this zone is mainly a passage-way allowing the lymphocytes arising in the cortex to reach the circulation. Finally, since lymphocytes are released from the thymus into the circulation, this organ appears to be composed of true lymphopoietic tissue, which participates in the formation of blood lymphocytes.

Plasmocyte formation in lymph nodes

Plasma cells—or to use present-day nomenclature, plasmocytes—fill the medullary cords of mediastinal lymph nodes in laboratory rats. It was therefore decided to apply to this material the same method as was used for thymic lymphocytes, in the hope of obtaining data regarding plasmocyte formation (Sainte-Marie, unpublished).

The nuclei of plasmocytes appeared to be quite similar to those of lymphocytes and showed approximately the same range in diameter variation. It was therefore decided to classify them into three types: a cell with large-size nucleus, referred to as large plasmocyte (plasmoblast); a cell with medium-size nucleus, referred to as medium plasmocyte (proplasmocyte); and a cell with small size nucleus, referred to as small or mature plasmocyte (Fig. 11). The nuclear diameters used above to classify the three types of plasmocytes were exactly the same as those employed above to classify the three types of lymphocytes. The cytoplasm of all plasmocytes stained a more purple hue than that of lymphocytes and, at least in medium and small plasmocytes, was far more abundant than in the corresponding lymphocytes.

It was soon found that each one of the three types of plasmocytes is capable of dividing and indeed mitoses were numerous. When the numbers of resting and dividing plasmocytes of the

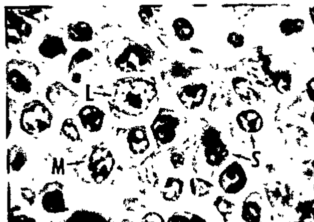


FIG. 11 [illegible]
node [illegible]
cytes a [illegible]

three types were counted, the mitotic index was found to be 7·22, 7·26 and 1·37 per cent respectively for large, medium and

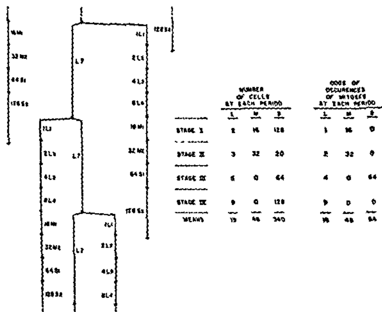


Fig. 12. Simplified model representing plasmocyte formation in medullary

tissue

small plasmocytes. These figures indicate rapid renewal of the three types.

The counts of cells and mitoses of plasmocytes made it possible to carry out the same type of analysis as was done for lympho-

calculate "expected" ratios, which were compared to the experimentally derived ones (Fig. 13). A fair agreement existed between calculated and experimental data.

Interestingly enough, the pattern of plasmocyte formation (Fig. 10) was quite similar to that obtained for lymphocytes (Fig. 3). On the average, there would be four generations of large, two of medium and two of small plasmocytes. The last generation would be formed of mature cells no longer capable of division, which presumably migrate outside the medullary cords. The nature of the stem cell was not apparent and it was tentatively suggested in the diagram that it may be a large plasmocyte, but further work is required on the subject. Whatever the case may be, large plasmocytes would undergo a series of successive divisions yielding plasmocytes with progressively smaller and smaller nuclei

The results indicate that plasmocytes may be regarded as the product of a lineage separate from the lymphocyte series. At least the data provide an explanation for their formation which implies that, contrary to a frequently expressed opinion (Maximow, 1932), medium and small plasmocytes are not derived from small lymphocytes undergoing cytoplasmic hypertrophy.

General conclusions

Models are presented for lymphocyte formation (Fig. 4) and plasmocyte formation (Fig. 12) These models were elaborated on the basis of (1) several assumptions, the main one being that all cells of a given morphological type behave in the same manner (same lifespan, same duration of mitosis, etc.); (2) experimental data consisting mainly of counts of cells and mitoses. Therefore the value of the models depends on the validity of the assumptions as well as on the accuracy of the experimental results. The very fact that it was possible to build coherent models, in good agreement with the data, is felt to be encouraging.

cytes. As a result, a pattern for plasmocyte formation in the medullary cords of mediastinal lymph nodes of normal rats was

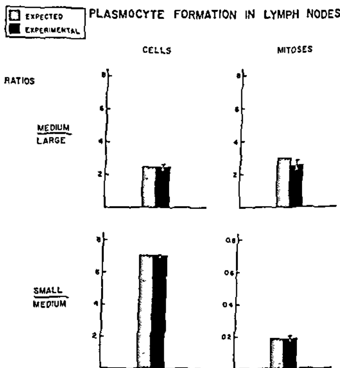


Fig. 13 Comparison between the experimental ratios obtained for the number of plasmocytes and their mitoses in the medullary cords of thoracic lymph nodes (dark columns) and those expected from the model in Fig 12 (shaded columns)

The values of the expected ratios are within the standard errors of the experimental ratios except in the case of the mitotic counts of medium over large plasmocytes, where the expected ratio is near the borderline of the error (Finally, the ratio of cell counts of small over medium plasmocytes is given for information only, since the method of calculation precludes its being used in support of the validity of the model).

devised (Fig. 12). The means for the numbers of cells and mitoses derived from this scheme (Fig. 12, lower right) were used to

that in the thymus cortex large numbers of small lymphocytes can be found in division. There are several interesting differences between the cortex and the medulla in the thymus. The small lymphocytes of the medulla are not only more motile, they are also slightly larger than those of the cortex. They also differ in their sensitivity to radiation—the small lymphocytes in the cortex are considerably more sensitive than those in the medulla. Another interesting finding is that after very high doses of radiation there is a clear-cut distinction in the reticulum cells as well: with a dose of about 13,000 r. you can kill every reticulum cell in the cortex without apparently affecting any of those in the medulla. This is a quite remarkable difference between cells which morphologically and potentially appear to be exactly the same.

Leblond: They do show several morphological differences. In the cortex, the cytoplasm of reticular cells stains less intensely and its limits are less distinct than in the medulla. Although reticular cells divide at the same rate in both locations, they appear to give rise to large lymphocytes in the cortex, but do not seem to do so in the medulla. Finally, signs of degeneration of these cells are not seen in the cortex, but they are common in the medulla. In fact, the Hassall's corpuscles of the medulla, a structure that always intrigues histology students, seem to consist of reticular cells that aggregate and degenerate together (Sainte-Marie, G., and Leblond, C. P. [1958]. *Proc. Soc. exp. Biol. (N.Y.)*, 98, 909).

Incidentally, as shown in our paper, the lymphocytes in the medulla, but not those in the cortex, show signs of amoeboid movement. Nuclei of lymphocytes may show long finger-like or squirrel-tail-like processes, as was first observed by Dr. V. E. Engelbert in Toronto (1956. *Canad. J. Zool*, 34, 707). This was so strange that many interpreted her observations as fixation artifacts. However, Dr. Sainte-Marie and I convinced ourselves that well-fixed preparations of rat thymus showed nuclear processes. But we saw them in the medulla only. We interpreted them as indicative of amoeboidism and diapedesis.

Fichtelius What happens if you define your classes of lymphocytes in another way? I think you could perhaps build another model, quite as beautiful as your present one.

Comparison between the data dealing with the lymphocyte and plasmocyte series revealed two surprising similarities. Firstly, nuclei of the same size appear to be cytologically identical in the two cell species. Secondly, the models (Figs. 4 and 12) show the same number of generations of large (4), medium (2), and small (2) cells of the two lines. The main difference was that the lifespan of medium and dividable small lymphocytes is approximately half that of large lymphocytes, whereas all dividable plasmocytes have the same lifespan.

Nevertheless, the two series—lymphocyte and plasmocyte—appeared to be distinct and to evolve independently.

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DISCUSSION

Trowell: I have already been quoted as saying that in rat lymphoid tissues small lymphocytes never divide. I would like here and now to correct that earlier statement and to agree entirely with Prof. Leblond

the proposed model, but the possibility of the existence of four generations of reticular cells may be kept in mind and eventually may lead to the histological identification of several types of these cells.

Yoffey: To cheer you up, Maximow (1927. *In* W. von Möllendorff's Handbuch der Mikroskopischen Anatomie des Menschen, 2, Part 1, 232, Berlin: Springer) maintained very strongly that the reticulum cell could go quite a long way in the direction of being phagocytic and still remain pluripotential.

Trowell: We have also made a detailed cytological study of the rat thymus, like that of Prof. Leblond. I believe there are two distinct types of reticulum cell to be found on purely morphological grounds. I think one type consists of typical reticuloendothelial cells just like those in bone marrow and lymph nodes. But in addition there is a *totally different type which is epithelial in nature and origin and in fact* represents the remains of the original thymus epithelium from which the thymus develops. I think these two types can be recognized morphologically, and I suspect that the cells which Prof. Leblond calls "pink cells" are the ones I regard as epithelial. In confirmation of this, if small fragments of rat thymus are cultured in hanging-drops of plasma clot, besides the emigration of lymphocytes, which of course soon die off, there is an emigration of typical macrophages—these are the reticuloendothelial elements, as you would expect. But in addition, after two to three days *in vitro* there is an outgrowth of characteristic sheets of epithelium, or a tissue which most cytologists would immediately say was epithelial simply from the way it grows in tissue culture, not as isolated cells but in great sheets. You never get anything of this nature from the culture of any other haemopoietic tissue.

Braunsteiner. How can you distinguish the different cell types in mitosis, Prof. Leblond? I presume you can distinguish medium and small lymphocytes, but can you distinguish mitoses from medium and from small lymphocytes?

Leblond: We think so. Small lymphocyte mitoses are more compact and solid than medium lymphocyte mitoses. The chromosomes are closely packed. In contrast, mitoses of reticular cells show long thin chromosomes in a large space. In mitoses of large lymphocytes the chromosomes are somewhat more condensed, while the cytoplasm is basophilic. And so on.

Leblond: I believe you would obtain the same model, even if the cells were defined in another way. For instance, you might very well define the small lymphocyte in such a way that, of their two generations, only the second (the one that does not divide) would be recognized as small lymphocyte. The first one might then be known as medium lymphocyte. The overall pattern of the model should not be affected.

Fichtelius: M. A. Kelsall (1958. *Univ. Colo. Studies*, 4, 93) found that lymph nodes regional to the thymus contained more plasma cells than any other lymph nodes of the body. Are the thoracic lymph nodes you examined regional to the thymus?

Leblond: Yes, the lymph nodes which were investigated for plasma cells were located next to the thymus.

Braunsteiner: Do you get any phagocytosis of the reticulum cells in the thymus?

Leblond: We do. However, by keeping the animals in good health under controlled conditions of food and temperature, it is possible to minimize the number of pyknotic lymphocytes as well as the number of reticular cells showing phagocytosis As you know, any stress that induces over-secretion of adrenal steroids elicits extensive pyknosis of thymic lymphocytes, and a large amount of debris is taken up by reticular cells. These changes are only occasionally found if the rats are free of disease and grow at a normal rate.

Braunsteiner: Then I presume the reticular cells are phagocytizing cells too?

Leblond: Yes. It is possible that the reticular cells are not a homogeneous population and indeed there may be two kinds of them. One type has a pinker cytoplasm than the other. Maybe only one type can phagocytize.

Incidentally, Dr. N. J. Nadler at McGill has carried out a mathematical analysis of the data recorded in the papers published with Dr. Sainte-Marie on the thymus. His calculations suggest that there may be four successive generations of reticular cells. The "initial" stem cell would go through four generations yielding 15 large lymphocytes (L_1) and one new reticular cell or "initial" stem cell. This has not been published, since we feel that the data dealing with reticular cells are not abundant enough to make it safe to introduce such complication in

megaloblasts: prophase 6′ 18″ in basophils and 5′ 20″ in polychromatics, metaphase 27′ 15″ and 23′ 21″, anaphase 5′ 40″ and 10′ 22″, telophase 17′ 22″ and 22′ 15″ (total time of mitosis 56′ 35″ and 61′ 18″, respectively); *pernicious anaemia megaloblasts:* prophase 13′ 21″ in basophils and 15′ 30″ in polychromatics, metaphase 40′ 15″ and 45′ 19″, anaphase 8′ 50″ and 11′ 03″, telophase 9′ 26″ and 14′ 36″ (total time of mitosis 71′ 52″ and 86′ 28″ respectively); (*d*) *human bone marrow normoblasts:* prophase 16′ 34″ in basophils and 17′ 06″ in polychromatics, metaphase 47′ 11″ and 54′ 29″, anaphase 11′ 49″ and 13′ 10″, telophase 16′ 28″ and 18′ 55″ (total time of mitosis 92′ 02″ and 103′ 40″, respectively).

Leblond: Can you tell us what the difference in duration of mitosis is like in the myeloid series ?

Astaldi: It is not very great. For the proliferative activity in the myeloid series the evaluation by the stathmokinetic test showed ratios from 5 to 2 and to 1, when passing from myeloblasts to promyelocytes and myelocytes. Regarding the mitotic time, for the granuloblasts considered all together Dr. L. Bussi reported 75 minutes, while Dr. Rondanelli for human bone marrow granuloblasts obtained a mitotic time of 112′ 49″, prophase being 21′ 30″, metaphase 65′ 16″, anaphase 14′ 42″ and telophase 11′ 21″. Moreover, for lymphoblasts Rondanelli gives the following times: prophase 12′ 25″ for lymphoblasts from chick embryo spleen and 15′ 02″ for lymphoblasts from human lymph gland, metaphase 33′ 11″ and 39′ 43″, anaphase 7′ 48″ and 10′ 18″, telophase 18′ 08″ and 22′ 20″ (total time of mitoses, 71′ 32″ and 87′ 23″, respectively).

Finally, let me mention the importance of the bubbling phenomenon at the time of cytodieresis. The cytoplasmatic bubbles, which during cytodieresis are continuously emitted and retracted, facilitate the cellular division in such a way that the time of division is shorter when [illegible]

Yoffey. If I remember aright, Dr. Astaldi, a generation time of about 19 hours has been found for chick megaloblasts (Salera, U., Tamburino, G., and Magnanelli, P. [1956]. *Progr. med.* (*Napoli*), 12, 677) and about 30 hours for myelocytes (Widner, W. R., Storer, J. B., and Lushbaugh, C. C. [1951]. *Cancer Res.*, 11, 877).

Astaldi: Do you think the duration of mitosis is the same in large, medium and small lymphocytes, and in the histiocytes too, or not?

Leblond: That was one of the assumptions, but it remains to be proved.

Astaldi: If the duration of mitosis is not the same in the different lymphocytic stages, you cannot compare the mitotic index of small lymphocytes with that of large lymphocytes, and so on. The mitotic index derives from the duration of the resting stage, as well as from the duration of the mitotic stage. Thus, the mitotic index may vary not only because of the variation of the interkinetic time, but also because of the variation of the mitotic time.

Leblond: The best way to show whether or not the model is correct would be to follow the behaviour of a large lymphocyte under living conditions, so as to see whether it yields the large progeny indicated by the model. Ideally, it would be nice to culture a single large lymphocyte. Could it be done?

Astaldi: Perhaps you can overcome your difficulties by experimenting with the stathmokinetic test, getting the stathmokinetic indices separately for large, medium and small lymphocytes.

Leblond Yes, I know, but that is difficult in the thymus because of the destructive effect of colchicine on the lymphocytes (presumably as a result of the release of adrenocortical secretions harmful to the lymphocytes, following colchicine injection)

Astaldi: I mean the stathmokinetic test *in vitro*. Moreover, you can measure the time of mitosis directly on the cells surviving *in vitro*. If you explant a fragment of embryonic lymph gland or spleen, you can observe directly the single lymphatic cells in their migration area. You might also obtain help for a direct observation of the developmental mitosis by using the cells from a tryptic treatment of the tissue, after their sedimentation on the culture slide.

Results regarding the time for the development of mitosis have been obtained by my co-workers, Dr. E. G. Rondanelli, Dr. E Strosselli, Dr. P. Gorini, and others. In following by cine-camera developmental mitoses of cells surviving *in vitro* they have observed these times: (*a*) *chick embryo mesoblasts*· prophase 18′ 04″, metaphase 49′ 55″, anaphase 9′ 19″, telophase 4′ 19″ (total time of mitoses 81′ 37″); (*b*) *chick embryo*

phery of the cortex inwards until they reach the medulla, where apparently they become endowed with active amoeboid motion—a property which allows them to enter lymphatic channels and blood vessels.

Stohlman: Is the increase in percentage label and intensity label of thymidine of the small lymphocyte on days 1, 2, 3, 4, compatible with the generative scheme you presented?

Leblond: On the whole yes, but there are complications due to the difference in the extent of labelling of the various types of thymic cells, as pointed out in the discussion of Dr. Everett's paper. The data published by Dr. L. S. Kelly, who examined lymphocytes in thymus and blood after labelling with [^{14}C]adenine or [^{3}H]thymidine, are not inconsistent with the model.

Yoffey: We have all been talking about feedback mechanisms. To what extent might there be a cellular feedback from bone marrow to thymus? This possibility arises out of the work of Weymouth and co-workers (Weymouth, P. P., Delfel, N. E., Doell, R. J., Steinbock, H. L., and Kaplan, H. S. [1955]. *J. nat. Cancer Inst.*, 15, 981). They [illegible]

regeneration quickly returned to normal. This type of observation suggests the migration of small lymphocytes from marrow to thymus, where they either enlarge and proliferate, or else stimulate the larger thymic cells to proliferation There is other evidence that the thymus can take up cells from the blood, either marrow transfusates containing a considerable number of small lymphocytes (Urso, P., and Congdon, C C. [1957]. *Blood*, 12, 251) or lymphocyte suspensions (Ambrus, C M., and Ambrus, J. L. [1959]. *Ann. N.Y. Acad. Sci.*, 77, 445). If [illegible]

leave the thymus.

Leblond When large and medium lymphocytes migrate out of the thymus, particularly to the perivascular lymphatic spaces, they are

Leblond: Dr. Astaldi, your comments are most pertinent. Indeed, two main factors influence our results—the time between two successive mitoses in a given cell type, and mitotic duration. The reasoning used in elaborating the model takes these two factors into account.

Astaldi: It is a general problem and not a particular one for your observations. That is why, to overcome the precariousness of the mitotic index in judging the proliferative activity of a given tissue or cell strain, I tried to use the stathmokinetic test and to evaluate the mitotic time.

Leblond: To give you an example of the rôle of mitotic duration, the scheme as presented implies that the lifespan or generation time of the medium lymphocytes is half that of the large lymphocytes. This conclusion arises from the fact that the mitotic index of the medium lymphocytes happened to be exactly twice that of large lymphocytes. However, the inference can be drawn only if it is assumed that mitotic duration was the same for both cell types. If further work were to show that the mitotic duration of medium lymphocytes is twice that of large lymphocytes, the scheme would have to be adapted to the new data. Perhaps we may then come to the conclusion that the life of medium lymphocytes is longer than postulated in the first model. Such refinements will help in giving precision to the model. Hence, I hope that your collaborator will soon complete measurements of the mitotic duration of lymphocytes. The question arises, however, whether estimates of mitotic duration on cultured cells yield the same results as *in vivo*.

Construction of our model was done blindly at first. We started assuming that we knew nothing, except for the assumptions I mentioned, and by trial and error with simple calculations, we came to successive conclusions, dealing first with the thymic cortex and later with the medulla. One of the conclusions was that small lymphocytes spend a longer time in both locations than other cells.

Everett: Could you define what you mean by "long time"?

Leblond: Not very well In our articles, this was defined relatively. For instance, in the peripheral cortex small lymphocytes (S_2) spend a time equal to twice the generation time of medium lymphocytes. In the deep cortex this was about three times as long, and so on. The story implies that small lymphocytes move gradually from the peri-

you can sometimes get even the very next cell, the large lymphoblast, on occasion. The significance of that worries us from two points of view. The first is the one you raised: at whatever point a cell leaves the reduction pathway is its further development, wherever it goes, predetermined? If it is, then one of those large lymphoblasts, wherever it goes, is simply at the beginning of a cellular chain reaction, and if it finally has to go through all its divisions, it represents potentially 128 small lymphocytes somewhere else in the body. Or: are the cells, when they leave at different stages of the reduction pathway, in a more genetically plastic state? Are they more easily influenced and able to branch off into other lines of development? Are they capable of functioning as our hypothetical stem cell if they leave earlier on in the pathway? I do not know as yet of any evidence bearing on it. This is a vital and fundamental point. From what few references I can get from the Brookhaven work in the few allusions to labelled lymphocytes in bone marrow, it has been medium and larger cells in small numbers which they have seen. Dr. Everett, who has presented our own evidence on bone marrow work, found a cell which I think was a medium to large lymphocyte.

Everett: I would say medium or perhaps large-medium. With respect to division of these cells in blood, it would appear to me that in relation to the number of cells present in blood versus lymph, divisions are as frequent in blood in the rat as they are in thoracic duct lymph.

Lajtha: Dr Cronkite, you said that when you labelled 12-hourly for 22 days with thymidine you only got about 30 per cent of the small lymphocytes labelled. This is very important and very relevant to the problem, as during those 22 days one would expect practically all of [illegible]

become labelled. This may mean that a large proportion of the medium and large lymphocytes never turn into small lymphocytes; it may mean that small lymphocytes come mysteriously from an unlabelled precursor; or it may mean that some small lymphocytes have a very long lifespan indeed.

[illegible]

frequently seen to divide. Hence our suggestion that any lymphocyte which leaves the thymus before reaching the final stage of the model (S_2) will continue dividing until it becomes an S_2 cell.

Fichtelius: One of your other basic assumptions was that only small lymphocytes left the thymus, but now you are assuming that medium-sized lymphocytes also leave the thymus.

Leblond: The assumption that only small lymphocytes leave the cortex was made when performing the calculation necessary to build the model. After the model was finished, it was found that the number of medium lymphocytes in the medulla was such as to require that some of these cells also come from the cortex. It was then possible to estimate to what extent the departure of medium lymphocytes from the cortex would affect the calculations previously made using the cortex data. The change in the number of medium lymphocytes thus introduced turned out to be within the range of the standard error. Hence, migration of medium lymphocytes is not such that the model had to be modified (see footnote on page 913 in the article by G. Sainte-Marie and C. P. Leblond [1958]. *Proc. Soc. exp. Biol.* (*N.Y.*), 98, 909).

Craddock: Osgood has seen a few lymphocytes dividing in the blood I have never seen them personally.

Cronkite: I have never seen them in the blood.

Leblond: I have seen only one case of division in the blood. There might be a mitotic inhibitor there. In the various organs and tissues, however, there may also be a continuous series of eight divisions, as assumed in the model accounting for the thymus data.

Yoeffy: I am very glad I raised that point, because you have touched on what is possibly, in view of the thymidine work, becoming one of the key things that has been worrying our own group about lymphoid tissue. The actual number of mitoses does not matter but if we take your eight mitoses one of your early cells represents 128 final small lymphocytes. You have this orderly series of cells moving along what we have come to describe as the reduction pathway—we could not think of a better name for it, and there is undoubtedly a gradual reduction in cell size. When we give thymidine we can label at any point along this reduction pathway. Also cells can leave at almost any point, except possibly the primary reticulum cell, which I cannot recall ever having seen in lymph; but apart from that I get the impression that

you can sometimes get even the very next cell, the large lymphoblast, on occasion. The significance of that worries us from two points of view. The first is the one you raised: at whatever point a cell leaves the reduction pathway is its further development, wherever it goes, predetermined? If it is, then one of those large lymphoblasts, wherever it goes, is simply at the beginning of a cellular chain reaction, and if it finally has to go through all its divisions, it represents potentially 128 small lymphocytes somewhere else in the body. Or: are the cells, when they leave at different stages of the reduction pathway, in a more genetically plastic state? Are they more easily influenced and able to branch off into other lines of development? Are they capable of functioning as our hypothetical stem cell if they leave earlier on in the pathway? I do not know as yet of any evidence bearing on it. This is a vital and fundamental point. From what few references I can get from the Brookhaven work in the few allusions to labelled lymphocytes in bone marrow, it has been medium and larger cells in small numbers which they have seen. Dr. Everett, who has presented our own evidence on bone marrow work, found a cell which I think was a medium to large lymphocyte.

Everett I would say medium or perhaps large-medium. With respect to division of these cells in blood, it would appear to me that in relation to the number of cells present in blood versus lymph, divisions are as frequent in blood in the rat as they are in thoracic duct lymph.

Lajtha. Dr. Cronkite, you said that when you labelled 12-hourly for 22 days with thymidine you only got about 30 per cent of the small lymphocytes labelled. This is very important and very relevant to the [illegible]

become labelled. This may mean that a large proportion of the medium and large lymphocytes never turn into small lymphocytes; it may mean that small lymphocytes come mysteriously from an unlabelled precursor; or it may mean that some small lymphocytes have a very long lifespan indeed

Cronkite· I am inclined to agree with you, Dr. Lajtha, but I would also like to bring up the problem that what I call a small lymphocyte may be different from what other people call a small lymphocyte. It

is a difficult problem. All I have to do is juggle the diameter a little and I obtain a different percentage of labelling. Or one can look at a lymphocyte and say it is a small one on appearance alone, forgetting about diameter. The percentage labelling is again different. There is a real problem of semantics and consistency and I find it difficult to be 100 per cent consistent myself in repetitive study of the same preparation.

Everett: We cannot ignore the fact that labelled small lymphocytes do not live for many days in lymph and blood unless we admit that the heavily labelled ones are different from the lightly labelled ones. I agree, too, that the small percentages with which we are concerned remain to be explained. I think if we employ Prof. Leblond's scheme it is easy to see how we have this diminution of label in cells from the precursors to definitive forms. I am not arguing for a short or long lifespan, I am just reporting the data.

Yoffey: I think we have reached the stage where we have become so timid about making any kind of definite statement that we call cells "mononuclears" rather than "lymphocytes", and are prepared to restrict ourselves entirely to "mononuclears" in radioautographs even when we can get a parallel dried smear and make the conventional classification, including clearly recognizable lymphocytes whose identity has never been questioned until now. As for the origin of the small lymphocyte, three hours after giving thymidine you can get some quite heavily labelled small lymphocytes in blood and lymph, and the only possible precursor for those first heavily labelled small lymphocytes is a medium lymphocyte, because in the lymph gland itself we see very few small lymphocytes dividing, if any. Unless, therefore, one is prepared to maintain that the small lymphocytes are a self-perpetuating population one must derive them from medium lymphocytes. In the thymus there may be more mitoses of small lymphocytes than elsewhere, but in the lymph gland there are far too few mitoses of these cells to account for the heavily labelled small lymphocytes which appear three to four hours after thymidine, and I can see no possible origin for these other than dividing medium lymphocytes. In view of these facts, I feel that if anyone maintains that the origin of small lymphocytes is from any cells other than medium lymphocytes, the onus rests upon them to demonstrate this alternative.

Stohlman: It seems to me that if you give thymidine every 12 hours over a period of 20 days and still have a number of unlabelled lymphocytes circulating, these must have been born before the thymidine was started, provided that the label is sufficiently heavy so that you do not miss labelled cells. This would speak for a long-lived lymphocyte such as is suggested by Hamilton's data (1959. *In* The Kinetics of Cellular Proliferation, p. 151, ed. Stohlman, F., Jr., New York: Grune & Stratton) and Ottesen's data (1954. *Acta physiol. scand.*, 32, 75). Perhaps there is some death of lymphocytes in the thymus and in the lymph nodes with a high intranodal turnover, so that one gets a high proportion of labelled lymphocytes in the node but only a few eventuate in a small lymphocyte. This possibility has been suggested by Brecher (1959 *In* Physiologie und Pathologie der Leukozyten, ed. Braunsteiner, H. Stuttgart: Thieme).

Yoffey: You need not go so far as killing them all off. We do know that in regions of actively growing tissue you get a fair amount of either cell death, or possibly a certain amount of cell impairment. If you have some lymphocytes which are not quite as active as others, the actively moving ones will get out quickly, either into the lymph or by direct entry. The others just fall by the wayside and stay there, possibly for quite a long time. There is one very clear line of evidence from several workers that you have at least two different lymphocyte populations, one longer-lived, one shorter-lived. It does not follow that if a certain part of a population of lymphocytes is long-lived, the whole population is long-lived.

Cronkite: Many years ago Dr. Trowell performed some very nice studies on the radiation sensitivity of small lymphocytes. They degenerate rather rapidly—the maximum amount of pyknosis is about five hours after injury. If the critical target of the small lymphocyte has a small volume it may be quite sensitive to tritium and be destroyed by a single hit or so, whereas if other cells have a much larger critical volume they might be expected to be more resistant to tritium. In addition the rapid removal of injured lymphocytes may be masking an injury that is being produced on a larger scale than we have observed. In this event we may be grossly underestimating the injury by [^{3}H]thymidine to small lymphocytes This is a distinct possibility although rather distasteful.

Yoffey: You think you would get lymphocytes as rapidly removed as that without seeing any evidence of it in sections?

Cronkite: There is a little increase in pyknosis of the lymphocytes in the lymph nodes in animals receiving continued injections—a little more than seen in the control rats. However, if the evidence of injury were being removed before one looked for it one could give repeated injections of [^{3}H]thymidine and never detect its harmful effects in this sensitive cellular system unless the injection and sacrifice intervals were proper.

QUANTITATIVE INVESTIGATIONS ON THE LYMPHOMYELOID SYSTEM IN THYMECTOMIZED RATS*

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The lymphocytopoietic activity of the lymphoid organs in rats has been studied by Kindred (1940, 1942) by determining the number of mitoses in the lymphoid organs per unit of tissue volume, by Andreasen and Christensen (1949) using mitotic counts in suspensions of cell nuclei, and by Andreasen and Ottesen (1944, 1945) using radioactive isotope labelling of deoxyribonucleic acid (DNA). The absolute numerical data from Kindred's investigations and the relative numerical data from the other investigations mentioned all agree in pointing to the thymus as the most effective lymphocyte-producing organ in the rat. Similar observations have been made in other rodents in more recent DNA turnover studies (Schooley, Bryant and Kelly, 1959).

In interpreting these data on lymphocyte production it must, however, be remembered that the mitotic activity cannot be directly taken as an expression of the relative efficiency of the lymphoid organs regarding the output of lymphocytes. In the first place it is not known with certainty how many newly formed cells perish without ever leaving the organs, although Kindred's investigations (1942) have already suggested that it is a minor part, though different in the thymus and in the lymph nodes. Next, the data in young animals must be evaluated taking

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the growth rate of the organs into consideration; and, finally, in the DNA-synthesis investigations the possibility of lymphocyte migrations from parts of the lymphomyeloid system to other parts must be borne in mind.

Thus, for the reasons given, a high DNA turnover and a high frequency of mitoses in the thymus do not necessarily mean that many cells are being released from the organ. The question has even been raised whether newly formed thymus lymphocytes leave the organ to any appreciable extent at all. The present work is an attempt to answer this question by means of extirpation experiments. The influence of thymectomy on the output of lymphocytes from the thoracic duct and on the blood lymphocyte content has been used as an expression of the extent to which the thymus contributes to the total output of lymphocytes from the lymphoid organs. At the same time a quantitative evaluation of the possible effects of thymectomy on the other parts of the lymphoid system has been done, knowledge of this being a necessary basis for a correct evaluation of changes in the lymphocyte content of lymph and blood.

The much discussed problem of the existence of functional relations between the lymphoid and the myeloid systems makes it reasonable also to examine the effect of thymectomy on the lymphocyte population of the bone marrow. Additional knowledge of the fate of the thymus lymphocytes may be obtained in this way.

Material and methods

Young female albino rats of an average weight of about 70 g at the time of thymectomy were used in the investigations. Thymectomy was performed through a mid-line incision in the upper part of the sternum in ten rats, and for each thymectomized animal an animal from the same litter and of approximately the same weight was subjected to a sham control operation. The average amount of thymic tissue removed was about 140 mg.

In no case were remnants of thymus found at autopsy. One of the thymectomized animals did not gain weight satisfactorily after the operation; it was therefore excluded from the investigation.

About 65 days after the first operation—after preliminary blood sampling for red and white cell counts and differential white cell counts—cannulation of the thoracic duct in the neck was performed under ether anaesthesia. On account of the possible diurnal variations in the thoracic duct lymph flow and in the blood lymphocyte content, these interventions were made at the same time of the day. Lymph was collected for one hour and the hourly lymphocyte output was calculated from the collected lymph volume in mm.^{3}—measured by weighing and specific gravity determination—and the lymphocyte content per mm.^{3} of lymph. Immediately after termination of the lymph collection the animals were killed by bleeding through incision of the abdominal aorta; the blood collected was centrifuged and the serum pipetted off.

Femoral bone marrow has been used for the bone marrow investigations. In order to obtain sufficient quantities it was necessary to use marrow from both femurs. The femurs were removed as soon as possible after the bleeding, the epiphyses cut off, and the diaphysis carefully split lengthwise by means of a pair of fine bone scissors. Following this the bone marrow cylinder could be easily removed. With only minor modifications, the further procedure follows the technique described by Yoffey (1955) for quantitative examination of the bone marrow in guinea pigs, so only the main points of the method employed need be mentioned. The removed bone marrow is placed in a previously weighed amount of autogenous serum, sufficient to give a dilution of the bone marrow in serum of approximately 1/15. The serum plus bone marrow is then weighed. The bone marrow cells are suspended in serum by vigorous shaking for three minutes, which gives a satisfactory suspension with a minimum of cell destruction. The specific gravity of suspension and serum is

measured by means of the copper sulphate method. The volumes of bone marrow and of serum can then be calculated, and from this again the volume dilution of bone marrow in serum. The absolute number of nucleated cells per mm.^{3} of suspension is determined by counting in a haemocytometer after dilution with a solution of methyl violet and acetic acid in the proportion of 1:40. The number of nucleated cells per mm.^{3} of suspension is converted into the number per mm.^{3} of bone marrow by means of the volume dilution. The number of red blood corpuscles in the bone marrow is determined according to the same principle; Hayem's fluid is used here as a diluent.

The serum-film technique of Harris (1956) has been used for the production of smears in order to keep the number of damaged cells as low as possible. The smears have been stained by the May-Grünwald-Giemsa method. The differential counts were made on 2,000 cells from each bone marrow.

Finally, the following sections of the lymphoid system were dissected out and the amount of tissue in the following groups determined by weighing. cervical lymph nodes (including all lymph nodes in the neck), mesenteric, thoracic, brachial, and epigastric lymph nodes, Peyer's patches, and the spleen. Smears were made according to the technique described by Trowell (1952), by teasing out in a drop of autogenous serum glands representing the two biggest groups of lymph nodes as well as Peyer's patches, and the thymus from the sham-operated animals. The smears were stained with Mayer's haemalum, and the number of mitoses counted in 2,000 cells from each group.

Results

Table I shows the thoracic duct lymphocyte output and the number of lymphocytes in the blood in thymectomized and in control animals.

The hourly output of lymphocytes shows a decrease in the thymectomized animals. This decrease is due to a drop in the

Table I

WEIGHT OF ANIMALS, LYMPH VOLUME, LYMPHOCYTE CONTENT OF LYMPH, THORACIC DUCT LYMPHOCYTE OUTPUT AND BLOOD LYMPHOCYTES IN THYMECTOMIZED AND CONTROL ANIMALS

	Number of animals	*Weight in g. 65 days after operation*	*Lymph volume mm³/hour*	*Lymphocytes /mm³ of lymph*	*Lymphocyte output × 10⁸/hour/100 g. animal weight*	*Lymphocytes /mm³ of blood*
Controls	10	147±7 7*	370±26·2	26,618±4,070	6·4±0·7	10,932±935
Thymectomized	9	134±7 4	327±21·9	16,121±2,110	3·9±0·5	6,647±791

Table II

MEAN WEIGHTS IN MG /100 G ANIMAL WEIGHT OF GROUPS OF LYMPHOID TISSUE IN THYMECTOMIZED AND IN CONTROL ANIMALS

	Cervical nodes	*Mesenteric nodes*	*Peyer's patches*	*Thoracic nodes*	*Brachial nodes*	*Epigastric nodes*	*Spleen*
Controls	69·5±6 6*	81 2±8·0	50 6±5·4	13·9±2 2	13·9±1·1	12·5±0·9	287·5±31·1
Thymectomized	61 4±5·6	73·8±9·7	51 5±4·6	11·5±1·5	13·1±1·3	9·0±1·2	251·8 ±5·3

* Mean ±standard error

lymphocyte content of the lymph, the hourly lymph volume showing no significant changes. The lymphocyte output in the thymectomized animals amounts to about 60 per cent of the lymphocyte output in the control animals. The blood lymphocyte content shows a similar drop to about 60 per cent of the values in the control animals.

The weight of the other sections of organized lymphoid tissue 65 days after thymectomy will be seen from Table II, which also shows the weight of the corresponding sections of lymphoid tissue in the control animals. The mean weights stated are calculated on the basis of weight in mg. of the groups of lymphoid tissue dissected out, per 100 g. of animal weight.

A comparison of the mean weights of the lymphoid tissue in the two groups of animals seems to indicate that the removal of the thymus has caused a minor decrease of weight in the remaining groups of lymphoid tissue, *except in the Peyer's patches*. The decrease in average weight is apparently greatest in the spleen. However, none of the changes is significant—not even the decrease in weight of the spleen. Further, before removal of the spleens for weighing, a more or less effective exsanguination of the animals has been performed. This involves a considerable factor of uncertainty, making alterations in weight of the spleen an incorrect expression of proportionate alterations in the content of lymphoid tissue. In the following, therefore, the spleen has been left out of account

The lymphocytopoietic activity of the *remaining* lymphoid organs in the thymectomized animals has been evaluated by determining the frequency of mitotic figures in representative groups of these tissues. The determination has been made on smears of teased cervical and mesenteric lymph nodes and of Peyer's patches

On comparison with the frequency of mitoses in the corresponding groups of lymphoid tissue in the control animals (Table III), it appears that the mitotic activity in the lymphoid organs of the thymectomized animals is unchanged.

Table III

MEAN NUMBER OF MITOSES PER 100 CELLS IN SMEARS OF TEASED CERVICAL AND MESENTERIC NODES AND IN PEYER'S PATCHES IN THYMECTOMIZED AND IN CONTROL ANIMALS

	Cervical nodes	*Mesenteric nodes*	*Peyer's patches*	*Thymus*
Controls	0 060	0·065	0·085	0·685
Thymectomized	0 056	0 056	0 078	

Table IV

MEAN NUMBER OF NUCLEATED CELLS, CELLS IN THE MAIN CELL GROUPS AND DAMAGED CELLS PER MM^3 OF BONE MARROW IN THYMECTOMIZED AND IN CONTROL ANIMALS

	Lymphocytes	*Erythroid cells*	*Myeloid cells*	*Blast cells*	*Monocytes*	*Other cells*	*Unclassif cells*	*Damaged cells*	*Total nucleated cells*
Controls	307 007 ±38 000*	875·876 ±47·900	886·719 ±51·000	42·504 ±3·550	15·923 ±1·390	29·708 ±2·200	23·621 ±1·750	252·295 ±23·800	2,433·653 ±76·900
Thymectomized	295·427 ±72·900	845 977 ±51·700	948·010 ±85·600	41·965 ±4·700	13 010 ±1·600	32·858 ±3·800	22·025 ±2·700	245·422 ±26·700	2,444·694 ±108·500

* Mean ± standard error

Thus, thymectomy has caused neither significant changes in the amount of other organized lymphoid tissue in the organism, nor changes in the rate of cell renewal in these tissues—and therefore has hardly caused appreciable changes in the output of lymphocytes from these tissues. Nor, as shown below, did thymectomy cause changes in the lymphocyte content of the bone marrow. It can be concluded therefore that the deficit of lymphocytes found in the central lymph and in the blood of the thymectomized animals can be taken as a fair expression of the loss of lymphocyte output from the thymus.

For comparison, the frequency of mitoses in the thymus of the control animals is also stated in Table III; it amounts to about ten times the frequency of mitoses in the other lymphoid organs examined.

The results of the bone marrow investigations are presented in Table IV.

The morphological criteria used in the identification of lymphocytes will be mentioned later. The myeloid cell group comprises mature granulocytes and their precursors, the erythroid cell group all precursors of the red blood corpuscles. The group, "other cells", includes plasma cells, reticulum cells, and megakaryocytes; the last-mentioned cell type was rarely found. Transitional cell forms between lymphocytes and blast cells are included in the blast cells

The total number of nucleated cells per mm^3 of bone marrow is $2 \cdot 4 \times 10^6$ cells in both groups of animals. There is also a close correspondence between the two groups of animals regarding the absolute number of cells in the main cell groups. In all groups of cells the individual variation in the number of cells is biggest in the thymectomized animals. The differences between the mean values in the two groups of animals are, however, not significant.

As a result of the previous exsanguination, the blood content of the bone marrow is slight. The content of mature red cells in the bone marrow averaged 1×10^6 red cells per $mm.^3$ of bone

marrow. With 8×10^6 red cells per mm.^{3} of blood this means that the maximum contamination of the bone marrow with blood is 0·125 mm.^{3} of blood per mm.^{3} of bone marrow. Consequently, only an insignificant portion of the approximately 300,000 lymphocytes per mm.^{3} of bone marrow owe their presence to the blood content.

Thus, the present investigation did not reveal quantitative changes in the cell population of the bone marrow following thymectomy.

Discussion

As previously mentioned, Kindred's (1942) histological studies suggest that under normal conditions the rate of cell degeneration and cell destruction in rat thymus is slight, only amounting to about 15 per cent of the total production of cells. Nor do Sainte-Marie and Leblond (1958) mention a greater rate of cell destruction in their investigations on the cell production in rat thymus. When the question of the extent of cell release from the thymus has, nevertheless, arisen, this is because—in addition to theoretical considerations of the kind mentioned in the introduction—the results of various previous experimental investigations do not accord with the concept of the thymus as an important source of lymphocytes. Among these investigations reference may be briefly made to earlier extirpation experiments in rats. Sanders and Florey (1940) found a minor drop in the blood lymphocyte content after extensive extirpation of lym-

short duration, but a re-establishment of normal values after three to six weeks This is surprising considering that in the present investigation thymectomy alone caused a lymphopenia nine weeks after the thymectomy.

In recent investigations, however, Schooley and Kelly (1958)

studied the rôle of the thymus in lymphocyte production in rats by means of extirpation experiments, and found results which on certain points are in fair agreement with those presented here. The decrease in the thoracic duct lymphocyte output, however, was far more pronounced in their investigations, falling to 27 per cent of the values in the control animals, the decrease in the blood lymphocyte content being less pronounced. In addition they found that the thymectomy had resulted in a significant decrease in the weight of the cervical and the mesenteric lymph nodes, of approximately the same order of magnitude as the decrease found by Reinhardt (1945) in thymectomized rats. They conclude that part of the decrease in the lymphocyte output may result from the decrease in the amount of remaining lymphoid tissue. This undoubtedly partly explains the rather considerable difference in the decrease in lymphocyte output between their investigations and the present ones, where no changes were found in the amount of remaining lymphoid tissue. In reality, therefore, the essential difference between the two series of investigations is the difference in the effect of the thymectomy on the other lymphoid tissues. The most tempting explanation of this difference may be sought in the fact that the thymectomies in the experiments of Schooley and Kelly were performed on newborn rats six days after birth, and in the present experiments on rats aged from 50 to 60 days. This again may mean that the difference observed in the state of the remaining lymphoid tissue in the two series of experiments is in reality an experimental confirmation of Kindred's (1940) histological observations that in very young, but not in older rats, the thymus provides lymphocytes necessary for the growth of the lymph nodes. Or—in other words—that the lymph nodes in very young rats at least are important destinations for the thymus lymphocytes

The diminution found in the thoracic duct lymphocyte output and in the number of lymphocytes in the blood following thymectomy suggests that the thymus to an appreciable degree

contributes to the total release of lymphocytes from the lymphoid organs. It must be emphasized, however, that there are too many unknown factors in the life history of the lymphocyte to allow of further conclusions to be drawn from extirpation experiments regarding the exact extent of the thymic contribution.

The number of nucleated cells in the bone marrow in adult rats was found by Burke and Harris (1959) to be $2 \cdot 32 \times 10^6$ cells per mg.; Říman, Veselý and Seifert (1959) found $2 \cdot 05 \times 10^6$ cells per mg. in rats weighing 170 g.; Mantz (1957) found $1 \cdot 79 \times 10^6$ cells per mm.^{3}; and Osogoe and Awaya (1958) found $1 \cdot 81 \times 10^6$ cells per mm.^{3} of bone marrow. Considering the varying techniques employed in the investigations, these figures are in good agreement and are in agreement with the $2 \cdot 4 \times 10^6$ cells per mm.^{3} found here. As regards the lymphocyte content of the bone marrow, however, the agreement comes to an end. The percentage content of lymphocytes in the three investigations last cited amounts to 31·1, 24 and approximately 12, and other investigations give still other values. The mean percentage content of lymphocytes in the bone marrow of the control animals in the present investigation was 12·7. The discrepancies are not surprising; as is often emphasized they represent the difficulties encountered in the identification of the lymphocytes. The best account of the morphology of the bone marrow cells in the rat has been given by Harris and Burke in their 1957 paper. The criteria by which cells in the present investigation were classified as lymphocytes agree fairly well with their description of "lymphocyte-like" cells: round cells measuring 8 to 12 microns with a narrow rim of pale, blue, clear cytoplasm, and a reddish-umber, smooth nucleus with only slight clumping of the chromatin. Cytoplasm was often invisible or apparently located at one pole of the cell.

The problem of the identity, origin, and fate of the bone marrow lymphocytes has been extensively reviewed by Yoffey (1950, 1954, 1955), Yoffey and Courtice (1956), and Yoffey,

Hanks and Kelly (1958). Impressive experimental and clinical evidence has been collected in favour of the view that the bone marrow lymphocytes are extramedullary-formed cells being carried by the blood to the bone marrow, there to act as stem cells for myeloid and erythroid cells.

Comsa (1957) made differential counts on bone marrow from [illegible] changes in [illegible] present investigations no changes were observed in the lymphocyte content of the bone marrow. This suggests that the thymus *is not* an important source of bone marrow lymphocytes. Granting the assumption that the bone marrow lymphocytes really are haematogenous, this means that the bone marrow lymphocytes must be mainly derived from the lymph nodes and that the thymus lymphocytes have other destinations. The existence of such a difference in destination of the thymus lymphocytes and the lymph node lymphocytes—a difference which possibly expresses a difference in function—has been shown to be highly probable by the transfusion experiments of Fichtelius (1958*a*, *b*). Fichtelius found that syngenesio-transfusion of radioactively labelled thymus lymphocytes in rats caused highest activity in the spleen, while transfusion of labelled lymph node lymphocytes caused highest activity in the bone marrow. This supports the conclusion which can be drawn from the results of the extirpation experiments, that the thymus does not supply lymphocytes to the bone marrow to any considerable extent. It must be added, however, that Harris (1958) in his studies on guinea pigs recovering from whole body γ-irradiation obtained results which may be interpreted in another direction. He found that for a certain number of days after the irradiation a close relationship existed between the lymphocyte levels in the bone marrow and the lymphocyte content of the thymus. The pattern of recovery was similar in the bone marrow and in the thymus, but differed from that in the lymph nodes. One of the interpretations advanced to

explain this relation is that the thymus plays an important rôle in the repopulation of the bone marrow with lymphocytes after irradiation. He also points out a close morphological identity between the bone marrow lymphocytes and the thymus lymphocytes; a similar observation has been made by Yoffey (1955). The observations of Harris (1958) apply to animals recovering from irradiation, and are therefore no serious objection to the conclusion drawn above, that the thymus lymphocytes do not make an essential contribution to the lymphocyte content of the bone marrow.

Summary

The rate of lymphocyte release from the thymus was investigated by means of extirpation experiments in young adult rats The hourly thoracic duct lymphocyte output 65 days after thymectomy showed a decrease to approximately 60 per cent of control values. A similar decrease was found in the number of blood lymphocytes. Thymectomy did not cause changes in the amount of remaining lymphoid tissue, or in the rate of cell renewal in these tissues. Therefore, the deficit of lymphocytes in the central lymph and in the blood can be taken as an expression of the loss of lymphocyte release from the thymus. The lymphocyte content of the bone marrow did not change following thymectomy. This suggests that the bone marrow is not an important destination for the thymus lymphocytes

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DISCUSSION

Roylance: Did you say that you found difficulty in differentiation on morphological grounds between the erythroblasts and the lymphocytes?

Bierring: Yes. I think it may sometimes be difficult to distinguish between a lymphocyte and an erythroblast which has reached a degree of maturation where its size is equal to that of the lymphocyte.

Roylance: I would have thought this was a straightforward business. I may be biased, having seen mainly guinea pig marrow and only a few rat marrows, all stained with MacNeal's tetrachrome, but there seems to be little comparison between the lymphocyte with its small knuckle of cytoplasm and pachychromatic nucleus, and the erythroblast, whether with basophilic, polychromatic or orthochromatic cytoplasm, as in the erythroblast there is far more cytoplasm and the nuclear chromatin is more densely clumped.

Bierring: The appearance of the chromatin is the main point in the differentiation between the two cell types, but in my experience it sometimes is still difficult to distinguish with certainty between these two cell types.

Yoffey: We have recently turned our attention to rat marrow because of the very great differences between rat and guinea pig which have been described. We are now adding the Lepehne test to our Giemsa-stained smears and we get much clearer differentiation.

Reinhardt: I am interested in this matter of the response of the animal depending on the age at which the thymectomy was carried out. It appears that there may be a difference in the reaction of the lymphoid tissues to thymectomy, depending on the age at operation. Experiments by Schooley and myself, in which thymectomy was carried out at a very early age, appear to demonstrate a more appreciable effect on lymph node tissues than when thymectomy was performed at a later age. There is also the interesting point that the location of the thymus in the species that have been studied (cervical thymus in the guinea pig, thoracic thymus in the rat) does not appear to be a factor in the experiments which have shown a fall in thoracic duct lymphocyte output after thymectomy. Have you any feeling as to what occurs, regarding the contribution of the thymus to other lymphoid tissues at varying ages, to account for these differences in data?

Bierring No. My only experience is with animals of this age, in which I did not find significant changes in the weight of the remaining lymphoid tissues. I think the thymus probably is more active at an earlier age.

Yoffey L. Gyllensten (1953. *Acta anat.* (*Basel*), suppl. 18, 1) in his experiments pinpointed the same thing, and got a transient hyperplasia of lymphoid tissue in newborn guinea pigs, but it passed off.

Jacobson. Lest we get to the end of lymphocytes and no one mentions the problem of what controls their production, I would like to hear the opinions of anyone in this field

Yoffey· You are referring to the lymphocytosis-stimulating factor?

Jacobson· I am not referring to anything! I am only asking whether there is any feeling that lymphocyte production as a whole is under some sort of control, and if there are any ideas in this direction.

Gordon. There is no doubt that the endocrine system is implicated in

the regulation of lymphocyte production. The studies of Dougherty and White and others have demonstrated important relations between stress, the adrenocortical steroids and the status of the lymphoid apparatus More specifically, stress or 11-oxysteroids cause widespread destruction and diminution in the numbers of mitoses in the lymphoid tissues which are probably responsible for the accompanying profound lymphopenia. Contrariwise, adrenalectomy may result in a rise in the absolute weight of the lymph nodes and an increase in the numbers of circulating lymphocytes. More recently, Dougherty (1959. *In* The Kinetics of Cellular Proliferation, p. 264, ed. Stohlman, F., Jr. New York· Grune & Stratton) has called attention to the increase in the mitotic rate and mass of the lymphatic organs of stressed adrenalectomized animals. Here the removal of the inhibitory action of the adrenal cortex permits stress to evoke hypertrophy of the lymphoid system. Thus it would appear that the adrenal cortex is one of the prime regulators of the lymphoid apparatus both under normal and abnormal conditions. There is also recent evidence by Gyllensten (1959. *Acta path. microbiol. scand.*, 47, 243) that the thyroid hormone may be an important stimulator of the lymphoid tissue.

As mentioned by Prof. Yoffey, evidence is also accumulating pointing to the existence of lymphocyte-regulatory mechanisms outside the confines of the orthodox endocrine system. In my paper here I will report the existence of a leucocytosis-inducing factor evoked in the plasma of rats by repeated leucocytapheresis Both absolute lymphocytosis and neutrophilia are produced by this principle in intact rats.

Yoffey· D Metcalf (1956. *Brit. J. Cancer*, 10, 431, 442) claims to have

is lowered quite appreciably, nevertheless it does continue even though at a lower level. If you accept the lymphocytosis-stimulating factor I think you have to accept also that even in the absence of the thymus there is some other lymphocytosis-stimulating factor. So by implication there would be at least two factors In this room, only a week ago, Abraham White came out very heavily and decisively in favour of the thyroid as the specific lymphoid tissue stimulant.

Loutit: Harping on the theme that perhaps we could learn something from pathology, I wonder if the recent observations, all rather scattered but now being collected, are of some importance; namely that adult human tumours of the thymus are associated not only with myasthenia gravis, but also in a certain number of cases with erythron-aplasia. This looks like another myeloid-lymphoid relationship. Has anybody got a synthesis for thymic function which will take care of the lymphopoietic-stimulating factor in the newborn on the one hand and of the pathological manifestations of thymic tumours of older persons on the other hand?

Bierring: A few cases have been reported where tumours of the thymus were accompanied by conditions of marrow arrest.

Cronkite. The opposite situation also exists in various tumours, perhaps of the kidney, ovary and cerebellum, which are correlated with polycythaemia vera. I have never seen it myself, but I understand that in some the polycythaemia will subside after the removal of the tumour.

Yoffey: The question of the age relationship is important In the growing animal you need to provide stem cells not only to allow for the normal wear and tear of the circulation, but also because the blood volume is growing the whole time. At that period you need more stem cells, and it may or may not be coincidental but that is the period when the thymus is at its most active. Morphologically, the small thymocytes are the cells which are most like the small lymphocytes in the marrow. However, after thymectomy there does not seem to be any obvious fall in the marrow lymphocytes. These findings could be interpreted—if the stem cell is a lymphocyte—on the assumption that the marrow has got to have its lymphocytic stem cells at all costs. While the thymus is functioning it gets them from the thymus, and when the thymus is removed it still maintains its lymphocyte populations by getting them from elsewhere.

Hulse: Two cases have been reported (de Vaal, O. M., and Seynhaeve, V. [1959] *Lancet*, **2**, 1123) of children who had complete absence of white cells, having neither granulocytes nor lymphocytes, with absence of lymphocytes in the thymus as well, and yet they were producing red cells The children eventually succumbed of course, but they did not seem to need lymphocytes to produce their red cells.

Yoffey: They did not live for very long after birth.

Hulse: But they managed to produce red cells—it was the absence of white cells which killed them.

Yoffey: The prenatal picture was not known. It may be that the critical thing there was to decide when the white cells disappeared It is clear that if one could establish cases where you get a complete absence of lymphoid tissue and yet erythropoiesis proceeds normally, in those cases you could not have lymphocytic stem cells

Hulse: Whilst on with this subject it is perhaps relevant to mention the differences between my work and that of P. F. Harris (1956. *Brit. med. J.*, 2, 1032), following irradiation. Harris gave guinea pigs a dose of just under 200 r. and followed the return of cells to the bone marrow. In his experience the lymphocytes returned first, showing a large temporary peak, and this was followed by a resurgence of red cell and granulocyte precursors. I examined rats after three doses of irradiation, 100 r., 200 r., and 400 r., and in each case the erythroid cells reappeared well before the lymphoid cells The myelocytes definitely reappeared before the lymphocytes after 200 r., and reappeared at about the same time as the lymphocytes after 400 r. This may of course be due to a species difference such as you mentioned in your paper, Prof. Yoffey.

Yoffey: I am prepared to accept species differences as an explanation of many discrepancies, but I must confess that I find it hard to believe that there could be such a fundamental difference as that

Cronkite: Various pathological peculiarities often do give a key to fundamental processes. There is the very interesting disorder of cyclic neutropenia in children, with the granulocytes disappearing and simultaneously being replaced almost quantitatively by what appears to be a typical monocyte (Page, A. R., and Good, R A. [1957]. *A.M.A J. Dis. Child*, 94, 623). I have not had an opportunity to follow these children, but we are following another child who since birth has never had more than about 10 or 20 granulocytes. No granulocytopoiesis can be found in the marrow There is a persistent 60 to 70 per cent monocytosis which appears to compensate in part for the deficit of the neutrophils. These reciprocal relationships may provide a key to understanding interrelations between cell types.

Another observation in fatally irradiated dogs that were kept alive

by antibiotics and blood transfusions until the marrow regenerated spontaneously may be important. There is no evidence for lymphocyte regeneration preceding the marrow regeneration. Admittedly the studies are not complete but it appears that the dog and man regenerate marrow without a preceding influx of lymphocytes.

Yoffey: In the pathological domain the thing you find is that during the agranulocytic period, unless it is a very heavily toxic condition, the marrow piles up with lymphocytes and when granulopoiesis is resumed the marrow lymphocytes fall. You get a lot of these interrelated facts; perhaps the lymphocyte is not involved at all in any of the cellular relationships. Then you have to start on the whole problem of why the marrow needs its enormous lymphocyte population.

Cronkite. In the aplastic anaemias that I have observed, prior to regeneration, it is not a lymphocyte that one sees. The cells that are [illegible]

organ the remainder of the organ regenerates and replaces the deficient part. If you studied these animals for a longer period—many weeks or months after thymectomy—did this deficient production still exist, Dr. Bierring? Does the same phenomenon occur after partial thymectomy?

Bierring: I studied these animals nine weeks after thymectomy and the production is still low. Partial thymectomies have been performed in guinea pigs by L. Gyllensten (1953. *Acta anat.* (*Basel*), suppl. 18, 1), who found that in newborn guinea pigs it caused a hyperplasia of the lymphatic tissues, but this was a transient hyperplasia, as after about four weeks the tissues returned to their normal size.

ON THE DESTINATION OF THYMUS LYMPHOCYTES

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The contribution of the thymus to the general pool of lymphocytes

That large amounts of lymphocytes are formed in the thymus of young animals has been shown with mitotic counts by Kindred (1942), Andreasen and Christensen (1949), Osogoe, Monden and Ito (1957), Osogoe and Awaya (1958), Sainte-Marie (1959) and Leblond (1959) and others The intense lymphopoietic function of the thymus has also been demonstrated with isotope techniques of various kinds by Andreasen and Ottesen (1944, 1945), Fichtelius (1953), Fichtelius, Gisslén and Hassler (1957), Fichtelius and Hassler (1958), Lundin (1958), Cooper (1959), Schooley, Bryant and Kelly (1959) and others. All agree that large amounts of lymphocytes are formed in the thymus, and the observation that [illegible] Dustin, [illegible] is also [illegible] erning the quantitative question as to what extent lymphocytes formed in the thymus degenerate there and in what proportion they actually reach the blood Kindred (1942) calculated that about 90 per cent of the thymus lymphocytes entered the blood stream, but this figure is very uncertain and the variation in the morphological picture is great (Andreasen and Christensen, 1949; Andreasen, 1959).

The thymic contribution to the blood lymphocytes has been studied by thymectomy (Reinhardt and Yoffey, 1956; Schooley and Kelly, 1958), which was found to cause a clear diminution of the output of lymphocytes from the thoracic duct. This effect can be explained by the assumption that the thymus is an important source of thoracic duct lymphocytes, but, as is stressed by Yoffey, Everett and Reinhardt (1959), such an interpretation of their experiments is rendered hazardous for different reasons. It can for instance be argued that the thymus supplies some hormonal factor, such as the lymphocytosis-stimulating factor of Metcalf (1958).

Schooley, Bryant and Kelly (1959) showed with tritiated thymidine that the pattern of labelling of lymphocytes in the blood bears a much closer resemblance to that in mesenteric lymph nodes than to that in the thymus. Preliminary results where tritiated thymidine was injected into a series of thymectomized mice showed very little difference from the control animals in the temporal pattern of labelling (Schooley, 1959). These experiments thus speak against the conception of the thymus as an important source of blood lymphocytes.

The route taken by thymus lymphocytes, supposing they actually leave the thymus, has been a subject of discussion. Kindred (1942) postulates that half of them must enter the blood stream via the thoracic duct and the remainder through the right lymphatic duct. Sainte-Marie and Leblond (1958) suggest another route in addition: by diapedesis from perivascular lymphatic channels within the thymus into the enclosed blood vessels. Schooley (1959), using rats, cannulated the right lymph duct in the neck and the thoracic duct in the abdominal cavity before it enters the pleural cavity. The results would indicate that about 10 per cent or less of the total output into the blood stream from the thoracic duct and right lymph duct arise from lymphatic tissue within the thorax. Although this is not conclusive it suggests that very few of the thymus lymphocytes leave the

thymus via these lymphatics. There may, of course, be other lymphatic routes.

From this introduction it is evident that more work is necessary to decide to what extent the lymphocytes of the thymus contribute to the general pool of lymphocytes and by which route they leave the thymus—if they leave at all. In the rest of this paper these difficult problems will be avoided and it will simply be assumed that some lymphocytes do reach the blood. It is the fate of these "hypothetical" thymus lymphocytes that is the subject of the present discussion.

The fate of transfused thymus lymphocytes

The most direct method of studying the problem is by transfusing a suspension of thymus lymphocytes and subsequently tracing the transfused cells (transfusion of lymphocytes as a method of studying their function has been discussed by Fichtelius, 1959*a*). For the sake of surveyability these investigations are presented in tabular form (Table I).

The survey makes it clear that the results are uniform. In all homotransfusions carried out the transfused cells were traced to the *spleen* and *liver* 24 hours after transfusion. In all heterotransfusions the cells were recovered in the *liver* but *not* in the spleen.* The difference in behaviour between homo- and heterotransfused cells was suggested to be due to different immunological states of the donors (Fichtelius, 1953, 1957*b*). The homotransfused cells derive from donors with an acquired immunity to the same infections as the recipient, whereas in heterotransfusion the donors have been exposed to other antigens.

* In Keohane and Metcalf's (1958) experiments the recipients were not examined after a longer interval than five hours According to Osogoe (1950) a period of 24 to 48 hours must elapse before a considerable accumulation of lymphocytes occurs in the liver and spleen, even when suspensions are injected directly into the portal circulation Osogoe (1950) writes: "This fact suggests that the process is no longer mechanical, but characteristic of the lymphocyte."

This suggestion has received some support from the fact that immunization of donors influences the behaviour of the transfused lymphocytes in the recipient in heterotransfusion. Fewer *active lymphocytes find their way into the liver of the recipient* when the donor is immunized than when the lymphocytes come from non-immunized donors (Fichtelius, 1959*b*). Similar results were obtained after homotransfusion from donors sensitized to dinitrochlorobenzene (DNB), which gives rise to the delayed type of allergy (Fichtelius, 1959*c*). Fewer of the thymus lymphocytes from sensitized donors were recovered in the spleen and liver compared to lymphocytes from non-sensitized donors.

Another observation which speaks in favour of some immunological function of the transfused thymus cells is their localization within the spleen of the recipient in the red pulp, particularly perifollicularly (Diderholm and Fichtelius, 1959*a*; Diderholm, 1960). The plasma cell proliferation during antibody formation takes place on this same spot (Huebschmann, 1913; Bjørneboe and Gormsen, 1943; Fagraeus, 1948; and others). Many research workers maintain that lymphocytes can be transformed into plasma cells (Roberts, Dixon and Weigle, 1957; Kelsall and Crabb, 1958; Schooley, 1959; and others). In this connexion an observation by Kelsall (1958) is very interesting: the regional lymph nodes of the thymus contain more plasma cells than any other lymph node. The present author's working hypothesis since 1953 has been that thymus lymphocytes normally settle in the spleen, there giving rise to antibodies during their transformation into plasma cells.

The old problem of the identity of the thymus and lymph node lymphocytes has been attacked with the aid of transfusion experiments. Lymphocytes of both types, labelled with ^{32}P, were transfused from rat to rat. There was a distinct difference in behaviour between the two types of cell, the thymus cells being chemically traced to the liver and spleen and the lymph node cells to the liver and bone marrow (Fichtelius, 1958*a*, *b*). Similar results were

Table I. A REVIEW OF EXPERIMENTS IN

Author	*Recipient animal*	*Donor animal*	*Label*
Osogoe and Hitachi, 1950	Rabbit, 2·5 kg.	Rabbit, 1·5 kg.	No label
Fichtelius, 1953	Rat, 3 months old	Rat, 3 months old	^{32}P *in vivo*
Fichtelius, 1953	Rat, 3 months old	Rabbit, 0·6 kg.	^{32}P *in vivo*
Karasawa and Osogoe, 1954	Rabbit, adult	Rabbit, young	No label
Fichtelius 1957*a*, *b*	Rat, 3 months old, with aseptic peritonitis	Rat, 3 months old	^{32}P *in vivo*
Fichtelius 1957*a*, *b*	Rat, 3 months old, with aseptic peritonitis	Rabbit, 0·6 kg.	^{32}P *in vivo*
Keohane and Metcalf, 1958	Rat, 150–200 g.	Rat, 150–200 g.	Acridine orange *in vitro*
Fichtelius, 1959*b*	Rat, 3 months old, immunized with *S. typhi* H antigen	Rabbit, 1·2 kg., not immunized with *S. typhi* H antigen	^{32}P *in vivo*
Fichtelius, 1959*b*	Rat, 3 months old, immunized with *S. typhi* H antigen	Rabbit, 1·2 kg., immunized with *S. typhi* H antigen	^{32}P *in vivo*
Fichtelius and Diderholm, 1959*a*	Rat, 3 months old, immunized with *S. typhi* H antigen	Rat, 3 months old, not immunized with *S. typhi* H antigen	^{32}P *in vivo*
Fichtelius, 1959*c*	Guinea pig, 1 month old, sensitized with dinitrochlorobenzene (DNB)	Guinea pig, 1 month old, not sensitized with DNB	^{32}P *in vivo*

WHICH THYMUS LYMPHOCYTES WERE TRANSFUSED

Method of identifying the lymphocytes	*Method of control*	*Interval after transfusion, and organs where lymphocytes were recovered*
Examination of histological sections	Injection of killed and injured cells	72 hours in the red pulp of the spleen, particularly perifollicularly and in interlobular spaces of the liver
Determination of [^{32}P]DNA	Injection of radiophosphate	24 hours. spleen and liver
Determination of [^{32}P]DNA	Injection of radiophosphate	24 hours· liver
Examination of histological sections	—	48–72 hours in the red pulp of the spleen, particularly perifollicularly and in interlobular spaces of the liver
Determination of [^{32}P]DNA	Injection of radiophosphate	24 hours· fewer active cells in the liver and more in the spleen compared to normal animals No transfused cells recovered as exudate cells
Determination of [^{32}P]DNA	Injection of radiophosphate	24 hours fewer active cells in the liver compared to normal animals Transfused cells recovered as exudate cells
Fluorescence microscopy	Injection of free acridine orange	5 hours. the cells were sequestrated and soon died in the lungs, liver, kidneys and spleen
Determination of [^{32}P]DNA	Injection of radiophosphate	24 hours liver, epidermis and bone marrow
Determination of [^{32}P]DNA	Injection of radiophosphate	24 hours: liver, epidermis? Fewer active cells in the liver as compared to the experiments with non-immunized donors
Determination of [^{32}P]DNA	Injection of radiophosphate	24 hours Liver and spleen More active cells in the liver and spleen as compared to normal recipients?
Determination of [^{32}P]DNA	Injection of radiophosphate	24 hours spleen and liver

Table I. A REVIEW OF EXPERIMENTS IN WHICH

Author	*Recipient animal*	*Donor animal*	*Label*
Fichtelius, 1959*c*	Guinea pig, 1 month old, sensitized with DNB	Guinea pig, 1 month old, sensitized with DNB	^{32}P *in vivo*
Diderholm and Fichtelius, 1959*a*	Guinea pig, 1 month old	Guinea pig, 1 month old	^{32}P *in vivo*
Diderholm, 1960	Guinea pig 1 month old	The same as the recipient (autotransplantation)	[^{35}S]methionine *in vitro*
Diderholm, 1960	Guinea pig, 1 month old	The same as the recipient (autotransplantation)	[^{3}H]thymidine *in vitro*

obtained in guinea pigs with radioautographic techniques (Diderholm and Fichtelius, 1959*b*) In the latter experiments, however, the lymph node lymphocytes were traced to the spleen as well, but to a relatively smaller extent than thymus lymphocytes. This difference in behaviour between the two types of cell is interpreted as functional, and is in keeping with the hypothesis of a relationship between thymus and spleen.

Attempts to trace thymus lymphocytes avoiding transfusion

The transfusion of labelled thymus cells from one animal to another is not an ideal method of ascertaining the fate of those thymus lymphocytes that reach the blood under physiological conditions. It is not known whether the lymphocytes that are normally released to the blood from the thymus are a special type

THYMUS LYMPHOCYTES WERE TRANSFUSED—*cont.*

Method of identifying the lymphocytes	*Method of control*	*Interval after transfusion, and organs where lymphocytes were recovered*
Determination of [³²P]DNA	Injection of radiophosphate	24 hours: spleen and liver. Fewer active cells in the spleen and liver than in the experiment with non-sensitized donors
Radioautography	Injection of radiophosphate and of heated, labelled cells	48 hours: in the red pulp of the spleen, particularly perifollicularly, non-follicular regions of the hepatic lymph nodes
Radioautography	Injection of [³⁵S]methionine and injection of labelled and heated cells	8 hours: numerous cells in the spleen perifollicularly Scattered cells in bone marrow, liver, lungs and lymph nodes
Radioautography	Injection of labelled and heated cells	8 hours: numerous cells in the spleen perifollicularly. Scattered cells in the liver and lymph nodes Bone marrow and lungs not yet examined

of cell. Osogoe and Hitachi (1950) and Keohane and Metcalf (1958) traced a mixture of all thymus cells, and Fichtelius (1953, 1957*a*, *b*, 1958*a*, *b*, 1959*b*, *c*) traced thymus cells which had synthesized deoxyribonucleic acid (DNA) during the labelling period of 24 hours prior to transfusion In the experiment with [³⁵S]methionine Diderholm (1960) labelled a mixture of all thymus cells, and in his experiment with [³H]thymidine, cells synthesizing DNA at the time of transfer were labelled. The transfusion experiments are unphysiological for other reasons, ... h relatively

... n labelling the cells *in vivo* and observing them within the animal that has formed them An attempt at "ideal" labelling was made by Diderholm and Fichtelius (1959*c*) Spleens of young rats were examined by radioautography at different intervals after a single

injection of ^{32}P. After six hours the blackening was mainly localized to the red pulp and small areas at the centres of the follicles. After four and eight days the appearance was different. The ... so th: ... is as follows. The blackening over the red pulp after six hours is due to thymus lymphocytes, which rapidly incorporate ^{32}P (Ottesen, 1954), and which have reached the spleen before the spleen's own lymphocytes in the white pulp have had time to become labelled. The fact that the radioautographs after six hours resemble the pictures obtained after transfusion of labelled lymphocytes supports this interpretation (Diderholm and Fichtelius, 1959*a*, *b*). If this interpretation is correct, thymectomy should influence the appearance of radioautographs of the spleen after administration of ^{32}P, the red pulp should be less blackened in thymectomized animals than in controls.

This was actually the case. Young guinea pigs were subjected to subtotal thymectomy or sham operation and were simultaneously given radioactive phosphorus (Fichtelius and Diderholm, 1959*b*). At different intervals afterwards the animals were killed and radioautographs made of the spleen. After four days the contrast between the white and red pulp was more striking in thymectomized animals. This may be due to absence in the red pulp of immigrant thymus lymphocytes. The results are not conclusive, however. Thymectomy may affect the spleen in other ways as yet unknown.

This investigation was, however, carried out with a quantitatively imperfect method, the quantifications being made subjectively. The series was also cut down since we planned similar experiments with better methods from a quantitative point of view. The influence of subtotal thymectomy on the radioactivity of deoxyribonucleic acid phosphorus (DNA-P) of the spleen was also studied in guinea pigs with a quantitative chemical method (Fichtelius, Diderholm and Stillström, 1960). The animals were

killed and examined four days after subtotal thymectomy or sham operation and injection of ^{32}P. No statistically significant differences were obtained. Thus the preliminary observations concerning the influence of thymectomy on the spleen could not be confirmed Either the earlier observations were due to chance (three thymectomized animals only were examined on the fourth day and three on the eighth day, and the method was not satisfactory from a quantitative point of view) or a real difference exists between the spleens of thymectomized and sham-operated guinea pigs. Further investigations with better methods are needed to establish the truth

In this connexion the question arises of what histological changes take place in the spleen following thymectomy. Subtotal thymectomy in newborn guinea pigs caused hyperplasia of all lymphatic organs with the exception of the spleen (Gyllensten, 1953). Hammar (1938) found lowering in splenic weight, reduction in red pulp, and reduction in size of the secondary follicles in thymectomized rabbits later immunized by intravenous injection of paratyphoid B, compared with intact controls; there was no significant difference in weight of the white pulp between the experimental animals and controls These findings are of interest in relation to Fagraeus's (1948) experiments *in vitro*, in which it was shown that cultures of red pulp always contained a considerably larger amount of antibodies than did follicular cultures. On immunization thymectomized rabbits, compared with intact controls, thus show a reduction in size of that part of the spleen that is most actively concerned with antibody formation, and that, according to the present author's hypothesis, is functionally related to the thymus.

In one of the transfusion experiments in rats thymus lymphocytes were chemically traced to the epidermis (Fichtelius, 1959*b*). In other transfusion experiments there was a similar tendency (Fichtelius, Danielsson and Hallander, 1960). The problem of the rôle of the thymus as a source of the lymphocytes in the skin was

also tackled with the aid of thymectomy (Fichtelius, Danielsson and Hallander, 1960). ^{32}P was injected into guinea pigs simultaneously with thymectomy or sham operation. After 25 days the animals were killed and the radioactivity of DNA-P of the skin was determined. This was significantly lower in thymectomized animals than in sham-operated controls. The result can be explained in the following way. Thymus lymphocytes are normally transported to the skin. By thymectomy the strongly labelled cells which should have been formed during the first days of the experiment are withdrawn from the skin. In sham-operated animals these heavily labelled cells are formed and within 25 days transported to the skin, thus giving a higher relative activity of skin DNA-P The incorporation of ^{32}P into skin DNA may, however, be lowered by thymectomy. A way of testing this is by injecting ^{32}P at various times after the operation and examining the activity of DNA-P after shorter periods of ^{32}P exposure, during which no transport of labelled lymphocytes has had time to take place. This was done in two experiments, which make it probable that ^{32}P incorporation into skin DNA is not affected by thymectomy Even if it can be shown, however, that thymectomy does not influence the incorporation of ^{32}P into skin DNA, the conclusion that thymus lymphocytes are transported to the skin is not safe. The results obtained can also be explained in another way. It may be that as a consequence of thymectomy the lymphocytes formed in the lymph nodes or elsewhere are diverted to other organs and reach the skin to a lesser extent than normally.

The thymus and antibody production

It now remains to be seen to what extent the hypothesis put forward on the relationship between thymus and spleen fits into known facts on antibody formation

The most direct means of proving that an organ plays a part in formation or storage of antibodies is probably the demonstration

of a high antibody titre within the organ after injection of antigen. In the spleen this was shown long ago (Pfeiffer and Marx, 1898; Wassermann, 1898). A higher antibody titre was demonstrated in the lymph nodes than in the blood (McMaster and Hudack, 1935). As far as the writer is aware, no one has yet succeeded in showing a raised antibody titre in thymus (Bjørneboe, Gormsen and Lundquist, 1947; Harris and Harris, 1954).

The spleen and lymph nodes have in common a profuse plasma cell proliferation during the formation of antibodies (Huebschmann, 1913; Bjørneboe and Gormsen, 1943; Fagraeus, 1948; Ehrich, Drabkin and Forman, 1949; and others). The rôle of the plasma cells in antibody formation has further been elucidated *in vitro* (Fagraeus, 1948). The thymus contains strikingly few plasma cells (Fagraeus and Gormsen, 1953; Yoffey and Courtice, 1956), and no increase in these cells has been observed to correspond with that taking place in lymph nodes and spleen under conditions of heightened antibody formation. Thus there are no grounds for assuming that significant antibody formation takes place in the thymus

By extirpating a given organ it is possible indirectly to demonstrate any rôle it may play in antibody formation; the antibody titre will then be lower than in control animals subjected to sham operation Experiments of this nature are hardly practical with lymph nodes, but the method has been employed with success for the spleen and thymus As long ago as 1892 Tizzoni and Cattani demonstrated a lowered titre in splenectomized animals Rowley (1950) found little or no antibody formation in splenectomized rats when small amounts of antigen were injected into the blood stream In contrast, when antigen was injected intraperitoneally, intradermally or intraportally, antibody formation was quite as marked as in normal rats. Antibody formation was not influenced by thymectomy when the antigen was administered subcutaneously (Harris, Rhoads and Stokes, 1948), but it was reduced after thymectomy when the antigen was introduced intravenously

(Hammar, 1938; Inzerillo and Chiti, 1953; Fichtelius, Laurell and Philipsson, 1960). In this respect, spleen and thymus show a striking parallelism.

It is thus highly improbable that antibody formation of any significance takes place in the thymus itself. Thymectomy experiments have shown, however, that the organ plays a part in this process. The effect must therefore be indirect. There is much to suggest that the factor promoting antibody formation is linked to viable cells, primarily lymphocytes.

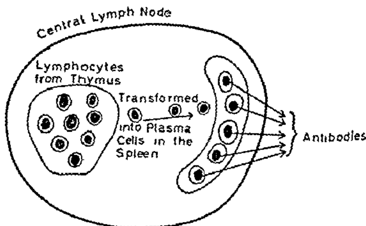


FIG 1. Thymosplenic integration in antibody formation.

Much work has been published concerning the passive transfer of antibodies and hypersensitivity by means of suspensions of viable cells from lymph nodes, thymus, and spleen (reviews of literature: Wesslén, 1952; Chase, 1953; Skog, 1956). Passive transfer of antibodies or of hypersensitivity fails, however, if the cells are damaged Fagraeus (1956) transferred antibodies passively with suspensions of cells from the spleen and from the thymus. The spleen preparation contained antibodies at the time of transfer, but none could be demonstrated in the thymus suspension at that time. Suspensions of lymph node cells have been

shown to contain antibodies (Harris and Harris, 1954). Thymus tissue produces tetanus antitoxin when transplanted into the anterior chamber of the eye of irradiated mice, and the transplants exhibit a "recall antibody response" when recipient mice are given an intravenous injection of the specific antigen (Stoner and Hale, 1955).

These findings concerning the thymus and antibody formation support the present a [illegible] the spleen may together [illegible]"— a "haemolymph node, if you will (Fig. 1). The thymus produces lymphocytes, and the spleen forms antibodies with their aid. Perhaps it is more than a chance that both organs appear at the same time in phylogenesis (Hartmann, 1930; Bargmann, 1943).

Summary

The formation of lymphocytes in the thymus is conspicuous, but it is not known how many of these cells leave the organ and how many die on the spot. Neither do we know whether the lymphocytes leaving the thymus are specially selected cells.

In a number of investigations ^{32}P-labelled thymus lymphocytes have been transfused from one animal to another. By a chemical method the transfused cells were traced to the spleen and liver of the recipient. The localization of the transfused ^{32}P-labelled lymphocytes within the spleen was studied radioautographically. The transfusion experiments have been followed up on the cellular level with lymphocytes labelled with [^{35}S]methionine and [^{3}H]thymidine.

All the transfusion experiments have certain inevitable disadvantages in common We do not know if the cells that are transfused are really representative of those leaving the thymus under physiological conditions. In certain experiments, among them comparisons between thymectomized and sham-operated guinea pigs, attempts have been made to trace lymphocytes within

the animal in which they were formed. The interpretation of these experiments is very uncertain, but they are more "physiological" than the transfusion experiments. They give some support to the hypothesis that some of the thymus lymphocytes are normally destined to the spleen, where they develop into plasma cells, possibly giving rise to antibodies in the process

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DISCUSSION

Yoffey: Professors Everett, Reinhardt and I found that with thymidine labelling, after four hours there was quite active cell production, if one accepts the normal criteria, in the germinal centres as well as in perifollicular areas.

The thing that worries us, looking at all this very interesting assembly of facts, is that it is hard to square up massive uptake by the spleen of cells from somewhere else, with the apparently very active formation of new cells in the spleen itself.

Fichtelius: We have used the method you mention, killing the animals one hour after the injection of thymidine. The slides from this experiment are now being exposed. We have also started an experiment where we give thymidine in a single dose and clamp the spleen artery for the next 30 minutes.

Lajtha: Dr. Fichtelius, you mentioned that you were not quite happy with the results with the transfused cells. I would heartily concur with that, especially in view of P. L. Mollison's findings (1958. *Brit. J. Haemat.*, 4, 115) that ordinary red cells when transfused (and very

much depending on the damage they received during pre-transfusion time) will be removed either by the liver or by the spleen. To some extent one could look upon transfused thymocytes as victims of a train crash, where some victims will be carried straight into the mortuary, and others into hospital, where they will subsequently die—or survive. Labelling experiments, with and without thymectomy, although definitely a better approach to the problem are not necessarily convincing. This is because a different pattern of uptake in the spleen of a thymectomized animal may mean a lack of cells transported from the thymus, but also may mean a different behaviour of spleen in a thy- [illegible]

and then release the cross-circulation and see what happens in that same animal both with and without thymus—the usual controls. In other words, allow the thymus and other cells to take up the label while the spleen is not in the circulation in that same animal, and just release the clamp of cross-circulation and see what happens.

Yoffey: The only trouble with a cross-circulation experiment is that it involves pre-operative stress and also the effect of the anaesthetic.

Lajtha: That could be checked with the controls.

Van Dyke: I have always been fond of parabiosis as a method for this sort of cross-transfusion I don't know whether it is applicable with thymus cells, because I don't know if there is any way of introducing the label into the thymus without labelling everything in the body. However, recirculation of lymphocytes could be very nicely studied using parabiosis and labelled thymidine because of the fact that with the very short biological half-life of the label in the circulation, there would be essentially nothing labelled in the non-injected partner. With such a system one could completely avoid all surgical procedures, heparin or any handling of the cells. The difficulty is in getting sufficient labelling of the lymphocytes of the donor (thymidine-injected) so that there are enough labelled cells to make accurate counts after they have been diluted in the recipient (uninjected member). This could be done by injecting labelled thymidine every 12 hours as described by Dr. Cronkite. In a few pairs which Dr. Schooley and Dr. Kelly have done, they found too few labelled cells in the peripheral

blood and thoracic duct lymph to make quantitative counts, but there were labelled cells in the thoracic duct of the non-labelled partner. With a higher percentage of the cells labelled, accurate quantification of the extent of recirculation could be made. Is there any way of introducing the label directly into the thymus by slow infusion through a cannula implanted in the thymus, using something like the stereotaxic instruments used by neuroendocrinologists?

Leblond: Local treatment has seldom been attempted. In one case, we noticed that hair follicles were intensely labelled at the site on injection of [^{3}H]thymidine but were only faintly labelled elsewhere. Hence, local injections may be worth trying.

Fichtelius: I have discussed (1959*a*, *loc. cit.*) the possibility of a mechanical sequestration of the thymus and lymph node cells as proposed by Mollison for the red cells, but this mechanism cannot explain all my results—for instance the difference between the behaviour of lymphocytes from immunized and non-immunized donors, and from sensitized and non-sensitized donors.

Lajtha: That would depend on the change of the surface of the cell.

Fichtelius: I have also found that hetero-transfused thymus lymphocytes can be transformed into peritoneal exudate cells in the recipients, and this cannot be explained as a mechanical process according to Mollison.

Gordon: Although marked atrophy of the thymus generally occurs in the aged animal, it is still capable of producing antibodies. There must therefore be some additional mechanism for supplying the spleen with antibody-producing cells. Have you taken this into account in your hypothesis, or do you believe that eventually the lymph nodes assume this function?

Fichtelius: I think lymph node lymphocytes can also settle in the spleen but in young animals the thymus lymphocytes settle there to a larger extent than lymph node lymphocytes.

Gordon: Then, in the aged animal, it would be the lymph nodes?

Fichtelius: Yes. And probably also lymphocytes formed in the spleen can be transformed into plasma cells.

Stohlman: In man the thymus is not as important an organ as it is in smaller animals. Also in man splenectomy does not affect the production of red cell antibodies to any extent. In considering some of these

things we should focus our attention on whether it is a general phenomenon throughout all species or relates more to one species than another. The same sort of thing holds true for the pituitary gland. In the rat hypophysectomy results in full-blown anaemia, whereas in man there is only a very modest anaemia. So we really ought to focus on which animal we are talking about.

Gordon: It should be borne in mind that hypophysectomized subjects are usually exposed to some form of replacement treatment.

Stohlman: This is without maintenance. We followed patients for about two or three weeks and red cell production was only very mildly depressed.

Gordon: You will recall that full-blown anaemia does not develop in hypophysectomized rats until later. In fact, Jacobson and his group have shown that the red cell mass does not decrease significantly during the first week or two following the operation.

Stohlman: You have to take into account the destruction. I am talking about production. In other words if you chronically irradiate a person and he does not develop thrombocytopenia for a long time, so that there is no superimposed bleeding, then it takes several weeks for anaemia to develop, but you can pick up changes in production long before that. The hypophysectomized animal used for erythropoietin assay develops maximum depression of production in about eight to ten days

Yoffey: As Dr. Stohlman has mentioned man, I feel that on behalf of my fellow anatomists and myself I must emphasize that in man, both before birth, and for several years afterwards, the thymus is a relatively very large organ

Leblond: In adults dying in automobile accidents, the thymus may be as large as or larger than in children. This fact is not well known, because in individuals dying of chronic illness in hospital the thymus is atrophic—and this is what pathologists see

Cronkite. I believe George Crile, Sr., in World War I performed autopsies on American soldiers and found that those who were killed quickly had large thymuses, and those who lay on the battlefield for a

size.

blood and thoracic duct lymph to make quantitative counts, but there were labelled cells in the thoracic duct of the non-labelled partner With a higher percentage of the cells labelled, accurate quantification of the extent of recirculation could be made. Is there any way of introducing the label directly into the thymus by slow infusion through a cannula implanted in the thymus, using something like the stereotaxic instruments used by neuroendocrinologists?

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Lajtha· Could an experiment be devised to check on whether there are enough plasma cells in the medullary loci of the lymph nodes you studied, Prof. Leblond? You could thymectomize the animal, shield a

veloped any accessory lymph node recovery. If it does occur in any significant amount, it obviates the necessity for any migration before plasma cell transformation.

Jacobson: Does anybody who has studied the transfusion of lymphocytes in the irradiated animal have any idea of the number that actually grew?

Yoffey: I don't know about growth, but you are probably familiar with the cross-circulation experiments of J. W. Hollingsworth, S. C. Finch and C. H. Chang (1956. *Blood,* **11**, 665), in which they found that the blood lymphocytes of an irradiated recipient rose almost to normal when it was connected with a normal donor, but disappeared in about 90 minutes after the cross-circulation ceased. But as far as I recall I don't think they followed the cells in any given organ. If the

Report No. 1193), working with Dr. Furth at Oak Ridge, transfused thoracic duct lymphocytes into irradiated animals, maintaining a constant normal level of lymphocytes, and obtained no radiation protection or regeneration in the marrow, presumably in animals that were probably genetically compatible, i.e highly inbred animals.

Jacobson Doesn't this seem strange? Certainly the experience has

Craddock In several instances we have transfused autologous *in vitro* [³H]thymidine-labelled lymphocytes back into dogs in a manner similar to the experiments Dr. Cronkite has mentioned We have been able to show just in peripheral blood studies the presence of labelled

Leblond: Dr. Fichtelius, has anyone done a quantitative estimation, perhaps by a radioactivity count, of the number of labelled lymphocytes present in various organs? You have repeatedly emphasized their presence in liver and spleen and I would like to know what the quantitative distribution elsewhere is like.

Fichtelius: My methods did not allow such calculations as you ask for—I have not even a rough idea of the amount of labelled lymphocytes in the liver and spleen after transfusion.

Leblond: It makes all the difference in the world to know whether a few or most of the labelled injected lymphocytes find their way into the spleen.

Fichtelius: I can add to the statement above that if the transfused cells settle in other organs examined the amount is too small to allow them to be traced with my chemical method.

Leblond: Has anyone attempted to separate labelled small and large lymphocytes by centrifugation or otherwise? This might be done by using cells from the thymus or cells obtained by cannulation of the thoracic duct. Tracing injected small or large lymphocytes might give different results.

Fichtelius: We are trying to separate large and small lymphocytes in the thoracic duct and the results are encouraging. The separation is carried out in the counter-streaming centrifuge constructed by Lindahl (Lindahl, P. E., and Nyberg, E. [1955]. *IngenVetenskAcad Tidskr.*, 26, 309). In the best separation we got 6 per cent of what we call large lymphocytes in one fraction and 35 per cent of these cells in the other fraction. We tried to separate thymus lymphocytes too, but we did not obtain as good results as with thoracic duct lymphocytes.

Leblond: In our paper presented here, it was mentioned that production of plasmocytes in thoracic lymph nodes of the rat may be satisfactorily accounted for by the proliferation of the large plasmocytes (plasmoblasts) found locally. Thus, it is not necessary to assume a lymphocyte metaplasia to explain plasmocyte production (nor does it seem that the transformation into plasmocytes is a method for the body to dispose of small lymphocytes). However, the possibility must be considered that the "initial" step in plasmocyte production is the transformation of a large lymphocyte into a large plasmocyte, which then through successive divisions would give rise to small plasmocytes.

large number of different media, from 199, Hanks', Eagle mixture, serum, lymph, etc., in all combinations, all pre-warmed at 37°. We took out the thymus and made the suspensions in about 32 seconds and irradiated immediately, followed by 10 minutes of incubation with the label. So far we have been unable to reproduce the *in vivo* sensitivity; the *in vitro* pattern appears straight away. Whether such damaged cells can recover eventually in the body if they are transfused, [illegible] n a [illegible] But

Cronkite: Some experiments by L. J. Cole, V. P. Bond, and others, rather crucially demonstrated this to me. As Dr. Lajtha has suggested, some casualties do recover. When homogenates of bone marrow or mouse spleen are examined by the phase microscope it can be seen that the cells have most of the cytoplasm ground off, yet they will protect against radiation injury It is with difficulty that one finds what looks like a really good intact cell. Therefore it looks as if these cells can reconstruct their cytoplasm and do a good job if given a little rest and a proper environment. This problem has been discussed in detail (Bond, V P, and Cronkite, E. P. [1957]. *Ann. Rev. Physiol.*, 19, 299).

Stohlman: I was going to bring up something along the same lines but with regard to red cells. If you damage red cells by storage, or freezing, etc., then on re-transfusion all the cells that are going to be lost will be lost within the first 24 hours, and survival of the remainder will be normal Apparently either the latter escaped damage, or if damage occurred it was repaired Dr. Fichtelius, have you any time curves on these thymocytes, on the rate of appearance of phosphorus in the spleen and liver after transfusion?

Fichtelius No. I have only examined the animals 24 and 48 hours after transfusion. As I said, it takes a certain time before these cells accumulate.

Stohlman. Are you sure it is the cells that are accumulating and not the phosphorus?

Fichtelius: When Osogoe and Hitachi (1950, *loc. cit.*) injected unlabelled thymus cells they found an accumulation of the cells perifollicularly. My ^{32}P-labelled cells gave blackening perifollicularly in the

lymphocytic cells for as long as four days after infusion. These are very few, but they are there without question, so I am certain that some of these cells survive and apparently proliferate.

Yoffey: You also got the radioactivity in the marrow in one of your experiments?

Craddock: That was not with tritiated thymidine but with DNA-^{32}P-labelled cells. The data there were no more striking for the marrow than for a variety of other organs, but we are not certain about that.

Loutit: We have not used the radioactive label for the lymphocyte but our usual chromosome label, so in order to ensure the survival of the irradiated animal we put in unlabelled bone marrow together with labelled lymphocytes. Then in the recovering animal we find unlabelled regenerating bone marrow, and partially unlabelled, partially labelled, regenerating lymphoid tissue. So some of these lymphoid cells—what proportion I have no idea—can and do recolonize lymphoid tissue. Again, we have no good time scheme on this, but it looks as if this label is progressively lost from the population of dividing lymphocytes. The later lymphopoiesis of unlabelled cells presumably comes from the stem cells provided by the myeloid tissues of bone marrow and splenic pulp.

Everett I want to emphasize the point Dr. Lajtha has made. In our transfusion experiments we administered cells labelled *in vitro* from the thymus, nodes and thoracic duct of littermate rats from a closely inbred strain. Invariably in our experience the very least *in vitro* manipulation makes quite a difference in the picture. A more physiological experiment, it seems to me, is the cross-circulation experiment where there is minimum handling and the operation is rapid. It is in those instances that we recover the largest number of tritium-labelled lymphocytes in thoracic duct lymph, bone marrow, spleen, Peyer's patches—none in the lungs.

Lajtha I would just like to cite an example of how these cells change functionally within a very short time We were intrigued with the great difference in the radiosensitivity of DNA synthesis of thymus *in vivo* versus thymocytes *in vitro*. With about 500 rads X-rays we get a 50 per cent depression of DNA specific activity, or of the mean grain counts *in vivo*, while *in vitro* we need something like 2,000 r. We tried very carefully to repeat the *in vivo* sensitivity *in vitro* by choosing a

it was the larger cells which produced the antibody, and not the smaller cells.

Cronkite: I want to amplify my earlier statement that when Cole, Fishler, and Bond (1953. *Proc. nat. Acad. Sci.* (*Wash.*), 39, 759) did this with the splenic homogenates it was the nuclear fraction that gave the protection. I had the privilege of observing these preparations. There were only fragments of cells that remained, yet they recolonized the marrow quite satisfactorily. So there is in all probability a repair potentuality for cells injured by homogenization.

Craddock. We have tried by a variety of techniques to study the cells that people are using for marrow transfusion after freezing, and these cells look very sorry by our criteria. In addition to this there are the experiments by people like A. Kornberg, using entirely synthetic systems of DNA synthesis except for a small portion of pre-formed DNA. In this system the cells can go ahead and elaborate an entire DNA molecule, which would certainly seem to suggest that the nucleus itself has some innate ability to develop itself without cytoplasm.

[illegible]

tion, but we tried this too, and found that if we used a DBA lymphatic leukaemia homogenate treated as he had treated the spleen cells we got tumour production. This latter approach has been criticised for the simple reason that after all a tumour may be virus-induced and therefore not good evidence that the injected homogenate had living cells.

The other amazing thing is that these days some people routinely use bone marrow which they take out, put in saline and use the next day. Obviously you can treat these cells, not with loving care, but with everything else, and they still seem to do well.

Lajtha: This raises the question: which is the cell which then recolonizes? There are certain cells which are tough, and which survive in extremely unpleasant surroundings—in fact they thrive on them. I have found certain reticular elements which live in a serum-saline mixture, with no change of medium for two or three weeks, where no cell should survive, and yet these are happy. Normoblasts, small

red pulp on the same spot. If you inject inorganic ^{32}P the blackening is over the white pulp, an entirely different location, and if you inject labelled but damaged cells you will have no blackening at all in the spleen. That is why we assume that it is the transfused labelled cells that give the blackening and in the tritiated thymidine experiments we also see the labelled cells intact within the spleen.

Leblond Are you sure the doses you used were such as not to cause damage? Damage produced with ^{32}P is extensive, as the cells appear to be very sensitive to the rays produced by that isotope. Some years ago, Dr. C. Stevens Hooper and I examined the location of ^{32}P radioautographically at various times after a single ^{32}P injection. At six hours, the red pulp was intensely radioactive. Soon after, there appeared instead a moderate reaction over it, which we first interpreted as being due to labelled cells migrating outward, but which turned out to be due to cell damage and death.

Fichtelius: In all my transfusion experiments I labelled the animal with 0 75 μc/g When I started the experiments I tried to find out if the cells were damaged. I found that more than twice as much ^{32}P did not decrease the lymphopoiesis and that the labelled cells were destroyed irrespective of their content of ^{32}P (1953, *loc. cit*).

Leblond· In the experiments in which I saw extensive lesions, 3 to 5 μc/g. had been injected into the rats.

Fichtelius· Roberts, Dixon and Weigle (1957. *A.M A. Arch. Path*, 64, 324) injected lymph node lymphocytes from immunized donors intramuscularly and subcutaneously in irradiated animals, and they found that the cells seemed to be transformed into plasma cells.

Yoffey· They also very convincingly described what seemed to be the formation of plasma cells in peritoneal exudates from something like a macrophage precursor But in general the earlier work, with fluorescent antibodies, of people like Leduc, Coons and Connolly (1955. *J exp. Med*, 102, 61) does show that antibody formation starts off in the primitive reticular cell The current view is that by the time you reach the mature plasma cell there is relatively little active new formation of antibody occurring

F. J. Keuning and L. B. van der Slikke (1950. *J. Lab. clin. Med*, 36, 167) tried to fractionate large and small lymphocytes, by differential sedimentation, in the spleens of animals forming antibodies, they found

445). They also find transfused lymphocytes settling down in large numbers in the thymus and so on. There is also a continuous drift of lymphocytes into the connective tissues. If intestinal elimination is the fate of the majority of the lymphocytes we must start thinking all over again about lymphocyte function.

Astaldi. Have Dr. Bierring and Dr. Fichtelius any ideas about the loss of lymphocytes through the intestinal tract in controls and thymectomized animals?

Bierring: No. I have been attracted by the problem and noted, as was also mentioned by Kindred, the large amounts of lymphocytes in the intestinal epithelium. It has been suggested, for instance by Andreasen, that they there act as some sort of generator for the renewal of the intestinal epithelium! To this, however, it must be added that [illegible]

Fichtelius In all my experiments I examined the intestine but I could never trace any lymphocytes to this organ. I have previously suggested that the intestine forms its own lymphocytes and does not depend on getting them from outside

Astaldi. In intestinal biopsies from humans, performed with Crosby's capsule, we have been able to show many lymphocytes which apparently crossed the mucosa and grew out in the lumen.

Fichtelius: I think the lymphocytes crossing the intestinal mucosa are formed in the lymphoid tissue of the intestine.

Astaldi: Yes, but what happens in the thymectomized animals as compared with the controls? Did you see any differences?

Fichtelius No

Trowell Warren Andrew, who originally described the presence of lymphocytes in the epithelium of the small intestine, said that most of the lymphocytes were within epithelial cells, and he found them both in the glandular epithelium and over the villi. The interesting thing was that those in the glandular epithelium never showed mitosis, whereas those in the villous epithelium showed very frequent mitosis. I would confirm that finding as far as my own observations go, at any rate in the rat and the cat. It is a very remarkable thing that one can find many small lymphocytes in mitosis in the epithelium of the villi,

lymphocytes or any other cells would be just blown to bits. So which is the cell that recolonizes?

Stohlman: The stem cell!

Jacobson: I am not going to settle that problem! But if we think of the erythroblast precursors as being stem cells, which we must at the present time, and if we reduce the bone marrow population of recognizable erythroblasts as far as we can determine to zero by hypertransfusion and keep it there for a long period of time, this bone marrow can then be transfused into irradiated animals and red cells are produced So that one has to assume somehow that one is dealing with a stem cell, and that it is tough and has a long life.

Astaldi. The results that Dr. C. C. Congdon showed last summer at the haematological meetings in Buenos Aires agree with the concept that stem cells carried in with bone marrow infusions are the cells which give rise to the repopulation after total body radiation injury

Bierring: I should like here to draw attention to the recent paper of J Berman and H. S Kaplan (1959. *Radiat. Res*, 11, 24) which shows that preliminary treatment of transfused isologous bone marrow, for instance with urethane or irradiation, does not alter the ability of the transfused bone marrow to repopulate the bone marrow of the irradiated animals, but it makes the bone marrow ineffective in promoting recovery of the thymus Those experiments therefore throw doubt on the view that bone marrow brings about thymic regeneration merely by repopulating the thymus of irradiated animals

Jacobson · How do you interpret these findings, Dr Bierring?

Bierring. Berman and Kaplan interpreted them as throwing doubt on the view that the ability to repopulate the bone marrow and the thymus does not depend on a common stem cell.

Jacobson But what does it depend on—two different stem cells?

Yoffey I don't know about lymphocytes recirculating but certainly theories about lymphocyte function recirculate and crop up periodically in cycles. One of the recent ones is this revival of the theory of intestinal elimination. Apparently the lymphocyte has quite random movement; we do not know of any attraction of any kind, chemotactic, biotactic or any other sort. C M and J L Ambrus have recently been working with Thiry-Vella fistulas, and have found lymphocytes in these intestinal loops in a short time (1959. *Ann. N.Y. Acad. Sci*, 77,

the vast majority of cells will die, certainly after the doses of radiation used. A small fraction will survive. Of that small fraction some may survive with a morphologically intact chromosome complement, others with a morphologically damaged chromosome complement. [illegible]

visible chromosome abnormalities which are still compatible with indefinite multiplication are, of necessity, very small. Therefore the chance in any one animal of finding the same morphological chromosome abnormality recolonizing spleen, lymph node, marrow, liver or [illegible]

same chromosomal abnormality?

Lajtha That is right—five different stem cells if you like. There will be many different types of abnormalities, but eventually you may be left with only one chromosome abnormality which is visible and still compatible with life.

Loutit: I cannot understand this thesis at all. Granted that most of the radiation-induced chromosome abnormalities seen with the ordinary light microscope seem to be incompatible with life, nevertheless in nature one finds numerous forms of chromosomal abnormality that are compatible with life The chance that any two chromosomal abnormalities could be identical in their morphological appearances seems to me to be so remote that we ought to regard any chromosomal abnormality as not quite unique but nearly unique.

Yoffey: We need a mathematician to start to calculate the probabilities.

Lajtha: Chance in itself would not exclude it. I quite agree that a visible chromosome abnormality of a particular type must be a unique phenomenon. I suggest, however, that of two visible chromosome abnormalities which are still compatible with existence, if one has a very small advantage of recolonizing by gaining just one mitosis, it can overgrow the other. I think Ford himself suggested that in his marrow-grafted irradiated mice the different clones can overgrow each other simply by stealing one single division time over the other.

and, apart from the thymus which we dealt with yesterday, as far as I know this is the only situation in the body where small lymphocytes can regularly be found in mitosis.

Astaldi: In human beings I have never seen mitosis in the lymphocytes of the villous epithelium.

Leblond: Observations with [^{3}H]thymidine in the mouse (Leblond, C. P., and Messier, B. [1958]. *Anat. Rec.*, 132, 247) showed a few labelled lymphocytes in the crypt epithelium and rare ones in the villous epithelium. In this regard, it may be emphasized that it can be readily shown with thymidine that lymphocytes do not transform into epithelial cells in the crypts as claimed by W. Andrew (1957. *Anat. Rec.*, 127, 457).

Yoffey: W. and N. V. Andrew (1949. *Anat. Rec.*, 104, 217) raised that point for two situations, for the intestinal epithelium and the basal layer of the skin, where the lymphocytes could be the precursors of epithelial cells

Lajtha: Dr. Jacobson asked a very basic question: are we dealing with one stem cell or two stem cells? Some experiments apparently indicate that you can recolonize bone marrow without recolonizing the lymphatic system, and *vice versa*. On the other hand, Dr. Loutit has mentioned the evidence for the recolonization of both the marrow and the lymphatic system with the same labelled clonal population. I should like to suggest that the experimental evidence is not contradictory, and in fact the concept that there are two stem cells would be favoured by the separate recolonization of marrow and lymph. The appearance of the simple labelled clone, as Dr Loutit described, could perhaps be due to the fact that after certain radiation damage only a very small proportion of the damaged population can survive—the vast majority of cells will die. It is very likely that survival is limited to a certain morphological chromosome abnormality which is still compatible with life. Therefore the possible abnormal chromosome morphologies which are still compatible with life must be very small indeed; consequently it is not necessarily surprising that both the marrow and the lymphatic system, if they recover, show the same chromosome abnormality

Loutit: I'm afraid I am a little confused.

Lajtha: The point is that when you irradiate a complex cell system,

the vast majority of cells will die, certainly after the doses of radiation used. A small fraction will survive. Of that small fraction some may survive with a morphologically intact chromosome complement, others with a morphologically damaged chromosome complement. We do know that most of the types of morphological chromosome damage are incompatible with life—the vast majority of the damaged cells will die after the first or second division. My thesis is that the visible chromosome abnormalities which are still compatible with indefinite multiplication are, of necessity, very small. Therefore the chance in any one animal of finding the same morphological chromosome abnormality recolonizing spleen, lymph node, marrow, liver or any other regenerating organ, may not be a small one, and it does not necessarily suggest a common stem cell.

Yoffey: You mean that two different stem cells could undergo the same chromosomal abnormality?

Lajtha: That is right—five different stem cells if you like. There will be many different types of abnormalities, but eventually you may be left with only one chromosome abnormality which is visible and still compatible with life.

Loutit: I cannot understand this thesis at all. Granted that most of the radiation-induced chromosome abnormalities seen with the ordinary light microscope seem to be incompatible with life, nevertheless in nature one finds numerous forms of chromosomal abnormality that are compatible with life. The chance that any two chromosomal abnormalities could be identical in their morphological appearances seems to me to be so remote that we ought to regard any chromosomal abnormality as not quite unique but nearly unique.

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Lajtha: Chance in itself would not exclude it. I quite agree that a visible chromosome abnormality of a particular type must be a unique phenomenon. I suggest, however, that of two visible chromosome abnormalities which are still compatible with existence, if one has a very small advantage of recolonizing by gaining just one mitosis, it can overgrow the other. I think Ford himself suggested that in his marrow-grafted irradiated mice the different clones can overgrow each other simply by stealing one single division time over the other.

Osmond: Two experiments Dr. Cronkite mentioned yesterday seem to give a good physiological approach to the problems of stem cell transfusion. The first was an experiment in which an animal could be protected from lethal irradiations by irradiating in two halves with a short time between. This would argue in favour of a quick migration of stem cells by the blood stream.

Cronkite: This work has been published in *Radiation Research* (Swift, M. N., Tabeta, S. T., and Bond, V. P. [1954]. *Radiat. Res*, **1**, 241). It looks like a piece of very clear-cut radiation protection. The upper half of the body was shielded and then as quickly as possible the shield was put over the lower half and the upper half was irradiated. Significant protection was obtained. These studies imply that the cell which protects must be in the peripheral blood migrating rapidly from one area to another in order to escape and be protected by moving the shield.

Osmond: Yet I understand that if one labels the upper half of the body, with the circulation to the hind limb occluded, and then releases the circulation, no labelling is found in the marrow cells of the hind limb. Is this only true in the normal case? Has the regeneration pattern been studied in such a hind limb after it has been irradiated?

Cronkite When the labelling takes place in the upper half of the body and the circulation to the lower half is occluded one can find labelled cells in the lower half after the circulation is re-established, but only in small numbers. It is easier to find the cells in the irradiated marrow but in either case a long search is needed. In the normal marrow large and medium-sized lymphocytes, a very rare labelled plasma-like cell, labelled reticulum cells, and, even more rarely, labelled promyelocytes are found. I do not think the latter can be considered a cell transformation since some get into the peripheral blood on rare occasions after stress. The number of these cells found in the irradiated marrow appears larger Whether it is the actual number that is larger, or whether they are not diluted by normal cells so that they are easier to find, is a problem that cannot be answered. Dr. Bond has found the same thing with parabiosis, cross-circulation and transfusion of labelled lymphocytes both in the normal and irradiated marrow

Yoffey P. Urso and C. C Congdon (1957. *Blood*, **12**, 251) showed that the depleted marrow picks up many more cells than the normal

marrow, as one might expect. How long after your labelling did you look for these cells in the marrow?

Cronkite: We commenced looking five minutes after re-establishment of the marrow circulation and continued for three days. Labelled cells were found as described earlier. However these are not perfect experiments. It is not possible to eliminate completely the circulation to the lower half of the body. Some blood leaks through the vertebral arteries. Abdominal compression and aortic clamping do not prevent this. However it is a very small fraction and probably does not label cells significantly since cells when found have an intensity of label similar to that in the upper half of the body.

Braunsteiner: If one tries to give a quantitative estimation of the need for stem cells there is a rather simple model. If you spleen-shield a mouse and give a lethal dose of 900 r. irradiation, then remove the spleen after about ten minutes, the animal is generally protected.

Jacobson: In our original experiments with spleen-shielding we observed survival even though the shielded spleen was removed 15 minutes after the irradiation-shielding procedure.

Braunsteiner: The cardiac output of a mouse is about 2 ml./min., and since only about one-fifth to one-tenth of the circulating blood passes through the spleen the amount which goes through in 15 minutes is probably not more than about 5 ml. In 5 ml. there might be about 25,000,000 mononuclear cells. In isologous transfer experiments 50,000 cells might be sufficient for radiation protection. Is this correct?

Jacobson: Our evidence indicated that some survival occurs in lethally irradiated animals even though as few as 25,000 to 50,000 embryonic blood-forming tissue cells are injected.

Braunsteiner: Twenty-five thousand is 0·1 per cent of 25,000,000. It might be sufficient therefore, if just about 0·1 per cent of the circulating mononuclear cells are stem cells.

Jacobson: I think that is about all it would require, and on the basis of the repopulation evidence I think this is the only explanation one could have.

Cronkite: I will make a statement for which I take full responsibility because Dr Bond and Dr Fliedner do not necessarily agree with me on this. The cells which circulate in the peripheral blood in normal man and that synthesize DNA are very few in number, about 10/mm.^{3},

and are divided into five general categories (see Bond *et al.* [1959]. *Acta haemat. (Basel)*, 21, 1). I believe that our type I is the most likely choice for the circulating stem cell.

Yoffey: Can you tell us what type I is?

Cronkite: A full description of this cell will be found in the paper. We do not call it the stem cell because my associates do not concur. There appear to be good grounds for eliminating the other four categories as stem cells, hence I prefer type I.

Jacobson: There is another thing that has to be taken into consideration in the spleen-shielding splenectomy experiments and the repopulation phenomenon. It is possible that in the manipulation procedure of spleen-shielding one actually squeezes out cells which ordinarily would not be appearing in the circulation in large numbers.

Loutit: Congdon and a collaborator did an experiment in which they pooled the buffy coat of the circulating blood of normal mice, and injected it into lethally irradiated mice: these mice died, but they have other mice in which they induced a leukaemoid reaction which was pretty polymorphic. This leukaemoid blood was used with success as treatment for lethally irradiated mice. Presumably there was a concentration of this stem cell, of type I or whatever it might be, in this leukaemoid blood, but again nobody has yet identified what it is.

Fichtelius. I agree with Dr. Bond and Dr. Fliedner, when they disagree with Dr. Cronkite. M Robbins (1959. *Amer. J. med. Technol*, 25, 87) has described different types of atypical lymphocytes. If you make a very thin smear there are more atypical lymphocytes and lymphocytes of different appearance than if you make a thick smear. It must be very hazardous to divide these mononuclear cells into five classes, because their appearance depends on the mood of the technician.

Yoffey Temperamental lymphocytes and stem cells are bad enough, but temperamental technicians !

PRODUCTION AND DISTRIBUTION OF GRANULOCYTES AND THE CONTROL OF GRANULOCYTE RELEASE*

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Department of Medicine, School of Medicine,
[illegible]

THE present work represents an approach to the kinetics of granulocytopoiesis which is an outgrowth of previous studies in this area. Much of the work is preliminary and is presented to give an idea of the direction of our investigation and thinking rather than established findings.

It is now well established that the normal myeloid marrow contains a mass of maturing granulocytic cells which provide a readily available supply of cells to be released to the periphery upon demand. This reserve of cells, which has been denoted as the marrow granulocyte reserve (MGR), is replaced about every five days in the normal human and releases mature cells in an orderly fashion and at a rate sufficient to replace the number calculated to be circulating in the peripheral blood on the basis of blood volume every few hours (Craddock, Perry and Lawrence, 1959).

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† This work was performed in collaboration with Drs. Seymour Perry, John S Lawrence, Leo Buxbaum and George R. Pieper, with the technical assistance of Miss Mary H Baker, Mrs Gloria Buchbinder, Mrs. Netty Buras and Mr Robert Yelenosky.

The granulocytes in the peripheral blood include those freely circulating (estimated from granulocyte count and blood volume) and those cells marginated in capillary beds. The size of this marginated group of cells is difficult to calculate and is probably constantly changing in different organs. Marginated cells are not a fixed separate pool, but are in a state of dynamic exchange with circulating cells. If one infuses normal, viable isologous granulocytes in large numbers (equal to or exceeding the calculated circulating mass), one observes either no elevation in cell concentration in the blood, or very transient elevation. If cell damage has occurred in the process of obtaining the granulocytes, leucopenia and an acute febrile reaction will accompany the infusion. If the cells are labelled they can be detected in the blood stream, but not in the numbers which would be expected if they were entering a closed system with limits defined by the circulating blood volume. If blood counts and determination of the concentration of labelled cells are carried out at frequent intervals during and after the cells are given, one observes irregular spikes with a falling concentration of labelled cells for 30 to 40 minutes. These fluctuations are not so marked if the cells are given slowly. It would appear that during this period of rapid changes in the circulating number of infused cells an equilibration with marginated cells is occurring. In our experience the total intravascular mass of granulocytes would appear to be between two and four times the circulating granulocyte mass in normal dogs. This compares favourably with calculations based on the data of Rosse and Gurney (1959), who employed morphological labelling by the cross-transfusion of normal subjects and patients with the Pelger-Huet anomaly. The actual number of cells marginated at any one time is highly variable and subject to changes in perfusion rate, cell adhesiveness, and factors influencing migration into tissue spaces (e g. bacterial endotoxins, inflammation, etc).

If cells are removed, labelled with as little trauma as possible, and reinfused, as in the work of Mauer and his associates (1959),

they disappear in an exponential manner with a half-time of seven to eight hours. It must be remembered, however, that much of the apparent disappearance soon after the infusion is due to equilibration of circulating and marginated cells, and that some of the labelled granulocytes can be detected for 48 hours and longer. These data agree with the findings of Rosse and Gurney (1959). In these latter observations no cell trauma incident to a labelling procedure was imposed. A relatively rapid turnover of blood granulocytes is also indicated by calculations of the rate of release of granulocytes from the marrow based upon the size and turnover time of the marrow granulocyte reserve (Craddock, Perry and Lawrence, 1959). It is of more than philosophical interest that the rate of production of granulocytes based upon these calculations of rapid blood turnover is still well below the daily production rate for erythrocytes.

Peripheral erythrocyte mass (70 kg man, blood vol. 5,850 cm^3, RBC $5 \cdot 0 \times 10^6/mm.^3$ or $5 \cdot 0 \times 10^9/cm.^3 \times 5{,}850\ cm.^3$) $= 29 \cdot 25 \times 10^{12}$ RBC

Erythrocyte survival = 120 days

Daily erythrocyte replacement rate, $\frac{29 \cdot 25 \times 10^{12}}{120} = 2 \cdot 44 \times 10^{11}$ RBC/day or $3 \cdot 5 \times 10^9$ RBC/kg./day

Peripheral granulocyte mass (70 kg man, blood vol. 5,850 $cm.^3$, gran count $5{,}000/mm.^3$ or $5 \cdot 0 \times 10^6/cm.^3 \times 5{,}850\ cm.^3$) $= 2 \cdot 92 \times 10^{10} \times 4$ (marginated cells) $= 11 \cdot 7 \times 10^{10}$

Granulocyte survival (average), 24 hours

Granulocyte replacement rate, 100 per cent/day
or $1 \cdot 17 \times 10^{11}$ granulocytes/day
or $1 \cdot 67 \times 10^9$ granulocytes kg./day

It is obvious that the range of error in calculations of this type is very wide. However, the significance of the comparison of erythrocyte and granulocyte production suggests that estimates of a very rapid peripheral turnover of granulocytes are not

inconsistent with the steady state productive capacity for haemic cells.

Another area of contention with respect to the circulation of the normal granulocyte is whether the cell re-enters the blood after migrating into the tissues. Evidence for this has not been forthcoming and there is a considerable bulk of data showing that this does not occur in normal conditions. It is necessary to differentiate re-entry of cells from tissue spaces from recirculation of cells marginated in the capillaries of lung or held within the sinusoids and pulp spaces of the spleen. It is also important to restrict this opinion of the lack of re-entry of cells to normal granulocytes and not to include immature cells of the granulocytic series, as in leukaemia. There is good evidence that red cells and lymphocytes may re-enter the blood via lymphatic channels. Leukaemic cells may be proliferating in foci throughout the body and discharging cells into the blood. Furthermore, as will be discussed subsequently, immature granulocytes circulate for longer periods than normally mature granulocytes. After the normal mature granulocyte leaves the vascular system, it performs its functions in the tissues, if called upon to do so. How long the granulocyte survives after leaving the blood would depend upon multiple factors but, in so far as the blood is concerned, the cell is lost once it leaves the capillary.

The most evident function of the granulocyte concerns its rôle in preventing bacterial invasion. The number normally circulating and harboured in various parts of the vascular bed and in tissue spaces is apparently more than adequate to maintain the defences against the host of bacteria normally in the gut, tracheobronchial tree, etc. A decrease in granulocyte number or the replacement of functionally adequate, mature granulocytes with immature abnormal varieties, as in the leukaemias, permits an overthrow of this important homeostatic mechanism. Under normal conditions the mature granulocyte is probably consumed quickly. When bacterial invasion threatens, the movement of

granulocytes into the inflamed area accelerates and this is compensated by acceleration of release of cells from the marrow granulocyte reserve. This arrangement permits a large number of mature cells to be called forth. It is probable that the mature

The experimental model of leucocytapheresis is appropriate for the study of the kinetics of granulocyte release from the MGR and the acceleration of granulopoiesis that occurs with this release. It has been shown that the release of cells in response to the increased peripheral removal of granulocytes, as in pyogenic infections, intravenous administration of bacterial endotoxin or creation of peritoneal exudate, follows the same pattern (Craddock, Perry and Lawrence, 1959). Schneiberg (1959) has recently obtained evidence for an almost immediate acceleration of cell production in response to the stimulus of bacterial pyrogen in humans. He performed careful quantitative cell counts, mitotic indices and coefficients on normal marrow during such a response and observed an increase in mitotic activity, first at the myelocyte level and later at the myeloblast and stem cell levels. We have attempted unsuccessfully on numerous occasions to detect a humoral factor in the plasma of leucocytapheresed dogs which would accelerate granulopoiesis. We have recently obtained some evidence that the control mechanism may involve the granulocyte itself.

Before describing these findings it is necessary to compare granulopoiesis in the normal to that in situations where the circulating granulocyte population does not consist of mature cells. Mature granulocytes are destined for rapid utilization after leaving the marrow. For this reason, the disappearance of granulocytes whose deoxyribonucleic acid (DNA) was labelled during their production and maturation in the marrow is rapid and the gradually falling curve of specific activity of the DNA

label in the blood granulocytes is a reflection of the diminishing concentration of the DNA label in the cells being fed into the blood as a result of mitosis of labelled precursors (Patt and Maloney, 1959; Bond *et al.*, 1959*a*; Craddock, Perry and Lawrence, 1959). Persistence of DNA labelled cells in the circulating blood, with a slower disappearance than normal, could occur for the following reasons:

(1) The granulocytes which are labelled in the marrow remain in the circulation for a longer time than in the normal steady state.

(2) The granulocytes are released prematurely or result from the division of precursor cells in the blood or tissues and hence are not held in a marrow maturation pool before appearing in the blood. In this situation the maturation of cells would occur in the blood, and if the immature cell is not utilized as rapidly as the fully mature cell the length of time it remains in the circulation will be prolonged, since it will include the maturation time which normally occurs in the marrow.

(3) The mitotic cycle of the proliferating cells is prolonged and hence the rate of dilution of the DNA label by division is slower than normal

(4) A significant percentage of the cells in the peripheral blood are so immature that they are similar to stem cells which will divide an infinite number of times and whose DNA, once labelled, will remain labelled to some extent as long as the cell remains viable. In this situation the amount of labelled DNA will be reduced by one-half (assuming they divide like normal stem cells) with each division, but after a large number of divisions the total reduction in label with each subsequent division becomes so small as to be undetectable

(5) Combinations of the above mechanisms.

If a dog is subjected to leucocytapheresis there is an acceleration in the entry of cells from the marrow and the granulocyte concentration in the blood rises rapidly after the procedure is terminated. As the blood granulocyte level flattens out the rate of release slows below normal, as shown by the rate of appearance

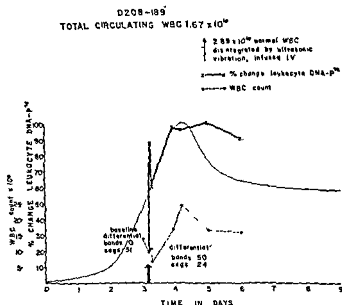


FIG. 1. The rapid release of [³²P]DNA labelled granulocytes from the marrow as part of the response to intravenous disintegrated homologous granulocytes obtained by leucocytapheresis. Note the left shift and the sustained plateau of DNA labelling

of DNA labelled cells. Nevertheless, the leucocytosis persists for 24 to 48 hours. This suggests that the younger forms, released in response to leucocytapheresis, remain in the circulation longer than fully mature granulocytes. This point is further supported by the type of data shown in Fig. 1. Here a marked leucocytosis was induced with the rapid release of "new cells" from

the labelled marrow. There was considerable "left shift". The fall in peripheral blood granulocyte [^{32}P]DNA specific activity

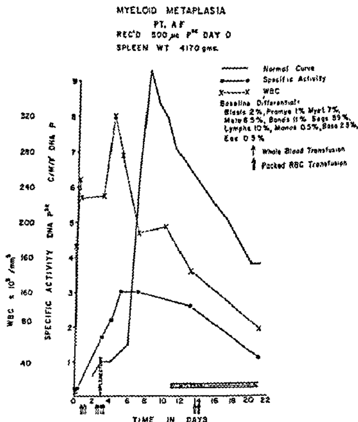


Fig. 2. The appearance and disappearance of [^{32}P]DNA labelled cells in the peripheral blood of a patient with myeloid metaplasia of the spleen and liver. Splenectomy on day 3 did not prevent the continued rise of the curve and did not result in a rapid disappearance of labelled cells.

is less rapid than normal, indicating that these immature cells circulate for a longer time, on the average, than the more mature population in the normal steady state. Additional evidence for this is provided by the fact that infections in humans or experi-

mental situations which are known to remove cells rapidly from the peripheral blood fail to cause a rapid fall in the curve of disappearance of DNA labelled granulocytes (Perry, Craddock and Lawrence, 1958).

Granulocytes which result from precursor division in blood or tissues without being held in a maturation pool in the marrow remain in the circulation for a longer total period than normal mature granulocytes, as shown by the prolonged disappearance of the DNA labelled cells in chronic granulocytic leukaemia in relapse, or myeloid metaplasia of the spleen, and leukaemoid reactions. Fig. 2 shows the appearance and disappearance of DNA labelled cells in a patient with myeloid metaplasia who required a splenectomy soon after the isotope was given. The peripheral blood showed considerable immaturity of the granulocytic cells. The spleen comprised a large portion of the total myeloid proliferative tissue and represented the major area of erythropoiesis, as shown by ^{59}Fe studies prior to splenectomy. In spite of the removal of this mass of proliferating tissue the specific activity of the DNA label in the circulating blood did not fall, and subsequently showed a slower fall than normal. This evidence [illegible]

mature granulocyte population The data in Fig. 3 show similar findings in a patient with a myelomonocytic type of leukaemia. Disappearance of the labelled cells was very slow until an infection supervened.

Patients with acute blastic leukaemia may show a marked prolongation in the disappearance of DNA labelled cells. Fig. 4 shows the data in a patient with acute myeloblastic leukaemia in the terminal blastic phase of the disease, resistant to the effect of 6-mercaptopurine and adrenal steroids. Prior to the *in vivo* DNA label being given, *in vitro* studies of the circulating cell population indicated a rapid and marked uptake of ^{32}P into DNA. From

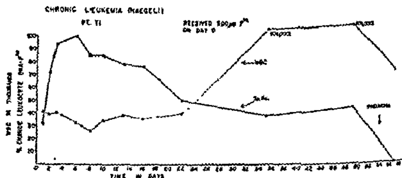


Fig. 3. The rapid appearance and prolonged disappearance of [32P]DNA labelled leukaemic cells in a patient with myelomonocytic leukaemia. Labelled cells remained in the circulation for a long period and finally disappeared at the time of a fatal pneumonia.

PT. E.K.
ACUTE MYELOCYTIC LEUKEMIA
SPECIFIC ACTIVITY DNA-P
TRANSFUSION OF WHOLE BLOOD
TRANSFUSION OF PACKED RBC
INFUSION OF NORMAL WBC
TIME IN DAYS

Fig. 4. Marked fluctuation in total circulating cell numbers in a case of myeloblastic leukaemia without acute alterations in the extent of [32P]DNA labelling in the circulating cell population.

[^{3}H]thymidine radioautographic studies it appeared that a high percentage of the cells in the blood were capable of proliferating (15 per cent labelled cells after three hours of incubation). Although definitive data as to the actual rate of DNA synthesis are lacking, it would appear to be very rapid since the specific activity of the [^{32}P]DNA was as high after three hours of incubation as after eighteen hours. Profound fluctuations in the concentration of leukaemic cells in the blood, associated with hyperuricaemia, resulted from the intravenous administration of normal granulocytes. The donors had received 0·2 μg. of a purified lipopolysaccharide endotoxin from *Salmonella abortus equi* (Pyrexal, courtesy of Wander & Co.) 24 hours prior to phlebotomy. This had been given to stimulate leucocytosis since, for the purposes of this experiment, it was desirable to obtain a high yield of granulocytes. These donor granulocytes failed to circulate, as indicated by the lack of fall in [^{32}P]DNA in the circulating labelled population. The pyrogenic reaction probably resulted from immediate release of endogenous leucocyte-endotoxin pyrogen. It will be noted that in spite of the marked fluctuations in cell concentration with destruction of cells, the time-course of the [^{32}P]DNA specific activity was not one of rapid fall. It seems likely that by the time these perturbations were induced, a large portion of the leukaemic population was labelled and the sudden loss of cells did not alter the concentration of the DNA label in the remaining population.

Fig. 5 shows similar data in another patient with acute blastic leukaemia Here the fall in total white count was spontaneous though possibly related to blood transfusion. The time-course of the [^{32}P]DNA specific activity appeared unaffected.

These data indicate the limitations of isotopic labelling of this type in the determination of cell survival in an immature cell population. It is clear that the same limitations prevail for any type of labelling of dividing cells unless the concentration of the label for each cell is determined.

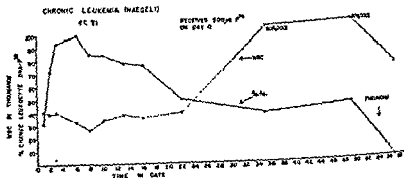


FIG. 3. The rapid appearance and prolonged disappearance of [32P]DNA labelled leukaemic cells in a patient with myelomonocytic leukaemia. Labelled cells remained in the circulation for a long period and finally disappeared at the time of a fatal pneumonia.

FIG. 4 [illegible] total circulating cell numbers in a case of myeloblastic leukaemia without acute alterations in the extent of [32P]DNA labelling in the circulating cell population.

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The data presented, in spite of their limitations, support the concept that the fundamental abnormality in leukaemias is a failure of cell maturation. The leukaemic cell, at least in the blastic leukaemias, lives for the purposes of division. The normal cell population comprises a small number of such cells which

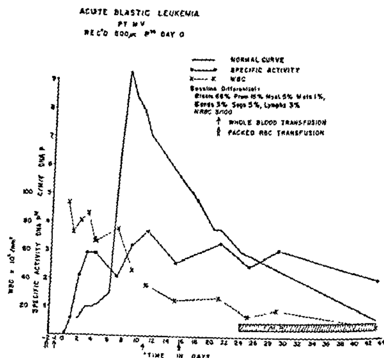


FIG 5. Rapid appearance and prolonged persistence of [³²P]DNA labelled cells in a case of blastic leukaemia in spite of a falling cell concentration.

procreate the maturing and mature cell population. All of the normal products of division are destined to die after a finite period of mature life. Leukaemic cells either divide or die. The ratio of immature proliferating cells to mature cells becomes steadily larger until it becomes the bulk of the cell mass. This increase in the bulk of cells which will either divide or die and which are

functionally defective is the hallmark of leukaemia. We clinicians are prone to think of the increasing mass of leukaemic tissue as being the result of accelerated growth and mitotic rate in the leukaemic tissue. However, as Lajtha (1959) has shown, the mitotic cycle of leukaemic blast cells may be slower than for normal haemic cells. The increase in bulk of proliferative tissue will occur, regardless of the rate of the phenomena of cell division, if maturation of cells and their utilization are impaired. It is clear that much additional study correlating mitotic activity, mitotic cycle time and cell turnover is necessary for elucidation of the kinetics of cell proliferation.

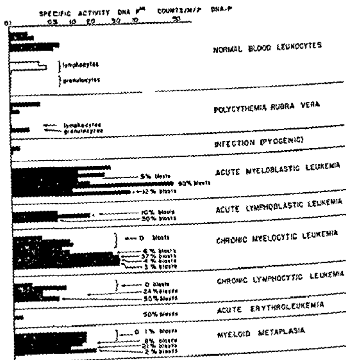
In vitro DNA synthesis rate of leukaemic cells as a function of cell growth

The leukaemic whose disease is progressing and not being held in check by therapeutic measures may flood his vascular system and tissues with immature leukaemic cells Some of these cells are prolife[illegible] DNA indicated [illegible] cultures. [illegible] subject of recent study (Bond *et al*, 1959*b*; Brecher, 1959). The systems we have used have consisted of suspensions of leucocytes or whole blood in buffered Hanks' solution-serum or lymph mixtures, the leucocyte concentrations being adjusted to approximately 10,000/mm^3, and with the addition of [^{3}H]thymidine, 0·25–1·5 μc per ml, and ^{32}P, 15 μc per 10^9 cells. Incubation times for both isotopes were 3 and 24 hours. Subsequently, it has become clear that a more precise estimate of DNA synthesis rate can be obtained by sampling at frequent intervals (every half-hour for three hours)

The results of three-hour incubations of leukaemic cells of different morphological varieties with the two isotopes are shown in Fig 6. In these data the term per cent cells labelled with

[^{3}H]thymidine includes only cells with heavy labelling (grain counts in excess of 15) unless otherwise specified. It will be noted

INCORPORATION OF P^{32} INTO DNA OF WBC
AFTER 3 HR INCUBATION WITH 15 μc P^{32}
PER 0.1 x 10^{10} CELLS



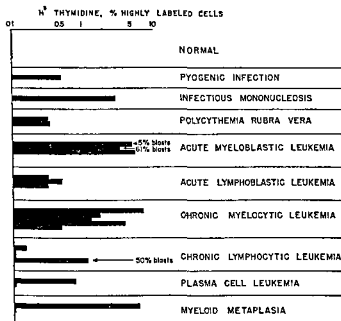
* Diagnosis of type of leukemia uncertain

(6a)

FIGS 6 a and b —The *in vitro* incorporation of ^{32}P and [^{3}H]thymidine by haemic cells Cells suspended in buffered Hanks' solution, pH 7.4, concentration 10,000/mm^3 Incubations at 37° [^{3}H]thymidine specific activity 1.9 c/m-mole, 0.5 μc per ml

that the results of the two methods of DNA labelling agree in general terms, and it has become our practice to employ both methods as checks against each other.

Several points are evident from these preliminary data. First, the extent of DNA labelling under these conditions does not correlate with the degree of morphological immaturity of the cell



(6b)

population. Thus, in the myeloid proliferative disorders, the highest degree of labelling was often observed in instances with a low percentage of blasts. Second, those conditions which were clearly lymphocytic, whether chronic or acute, showed very low uptake. This is of especial interest in view of the high degree of

labelling observed in normal thoracic duct lymphocytes and in the atypical lymphocytes of infectious mononucleosis. Two cases of plasma cell leukaemia with large numbers of blast cells in the blood showed very low DNA labelling.

If it is true that DNA synthesis occurs in proliferating cells only, and if it is assumed that this phenomenon correlates with the rate of cell division and intermitotic interval, it would appear that blastic leukaemias are not always associated with an acceleration in mitotic rate. Indeed, the mitotic cycle of these leukaemic blasts may be much longer than for the normal blood cell precursors, as shown by Lajtha (1959). The total bulk of proliferating tissue in relationship to the bulk of maturing and dying cells is probably more important in the overall course of the disease More evidence is needed, however, before it can be stated with assurance that the rate of incorporation of an isotope into DNA is a specific measure of either DNA synthesis rate or mitotic activity. For one thing, the uptake of the label may be obscured by the amount of unlabelled intracellular precursor. For another, cells which synthesize DNA rapidly may not divide subsequently or may do so in a delayed manner. More data correlating mitotic indices with DNA synthesis are needed in this regard.

We have studied the effect of different types of sera and plasmas in the incubation mixtures of immature cells and it is clear that they differ in their influence on DNA synthesis, but no consistent pattern has emerged. It has long been known that sera differ in their influence on cell growth in tissue culture (Osgood, 1959) It has also been shown that sera differ in their influence on nucleic acid synthesis (Thomas and Lochte, 1957, Thomas, 1959; Frenster, Best and Winzler, 1958) and it has been postulated that this may be due to an inhibitor(s) which is more apparent in normal serum than in that of leukaemic patients (Frenster, Best and Winzler, 1958). The possibility exists that this effect is due to differences in serum content of DNA precursors. The sensitivity

of [^{3}H]thymidine labelling of cell DNA to the amount of carrier thymidine is shown in Fig. 7.

Dog thoracic duct lymphocytes provide a source of proliferating cells of excellent purity and morphological quality for this type of study. There is no cell trauma incident to obtaining and separating the cells for study, and a significant percentage of the cells (4 to 15 per cent) are actively synthesizing DNA. The lymph also provides an excellent culture medium for incubation of the cells—a system which reproduces the *in vivo* conditions

% HIGHLY LABELED DOG LYMPHOCYTES H^3 THYMIDINE
1 2 3 4 5 6
CONTROL
LYMPH + THYMIDINE 0.001 mM/ml
LYMPH + THYMIDINE 0.01 mM/ml
0 LYMPH + THYMIDINE 0.1 mM/ml
LYMPH + THYMINE 0.001 mM/ml
LYMPH + THYMINE 0.01 mM/ml
LYMPH + THYMINE 0.1 mM/ml
0 LYMPH + THYMINE 1.0 mM/ml

FIG 7 The effect of addition of carrier thymidine and thymine on the uptake of [^{3}H]thymidine by dog thoracic duct lymphocytes (3-hour incubation in lymph)

very closely for lymphocytes As part of the study of DNA synthesis in these cells we have observed a peculiar effect of granulocytes. Mature canine granulocytes markedly inhibit the uptake of [^{3}H]thymidine and ^{32}P into DNA by lymphocytes when the two types of cells are present in the same incubation system. This inhibition is very marked, as shown in Fig 8, and in some instances it totally abolished uptake of the label after three hours of incubation. During and following such an incubation both varieties of cells appeared normal morphologically both on stained smears and on phase microscopy Vital staining characteristics and motility were not altered.

% HIGHLY LABELED CELLS, H^3 THYMIDINE

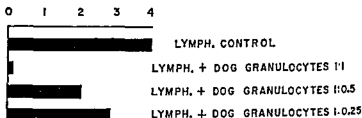


FIG 8. The effect of different concentrations of intact viable homologous dog granulocytes on DNA synthesis by dog thoracic duct lymphocytes.

% HIGHLY LABELED LYMPHOCYTES

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

CONTROL (LYMPH.)

0 INTACT NORMAL DOG GRANULOCYTES

0 HOMOGENIZED DOG GRANULOCYTES

0 HOMOGENIZED DOG GRANULOCYTES, DIALYZED

DIALYSATE, HOMOGENIZED DOG GRANULOCYTES

SALINE + LYMPH (1.1)

SALINE ELUATE + LYMPH

SALINE, BOILED

CONTROL

NORMAL DOG GRANULOCYTES, FROZEN, STORED

DOG LYMPHOCYTES, FROZEN, STORED

CONTROL

CELL FREE SALINE, AFTER 2 HR INCUBATION WITH NORMAL GRANULOCYTES

FIG. 9 Dog thoracic duct lymphocytes Inhibition of DNA labelling with [^{3}H]thymidine by granulocytes (3-hr incubation)

The granulocytes in these experiments were obtained by dextran sedimentation of peripheral blood from the same dog whose lymphocytes were being studied. In other instances granulocytes obtained by leucocytapheresis were employed. The inhibition was equally striking with viable, actively motile granulocytes and

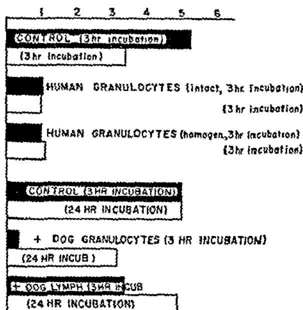


FIG 10 Granulocyte inhibition of thoracic duct lymphocyte DNA labelling with ^{32}P and [^{3}H]thymidine Specific activity [^{32}P]DNA, c/m/μg DNA-P □ % highly labelled lymphocytes [^{3}H]thymidine ■

cells disintegrated by homogenization, ultrasonic vibration or freezing and thawing (Fig. 9) Some inhibitory effect could be shown in saline extracts of such cells, but not as much as in the cell fragments. The inhibitory effect was not removed by dialysis overnight against water and did not appear in the dialysate. The inhibitor effect was found in the supernatant saline and Hanks'

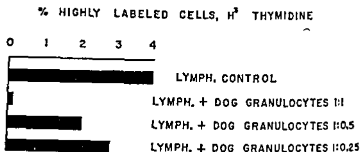


FIG. 8. The effect of different concentrations of intact viable homologous dog granulocytes on DNA synthesis by dog thoracic duct lymphocytes.

% HIGHLY LABELED LYMPHOCYTES

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

CONTROL (LYMPH)

0 INTACT NORMAL DOG GRANULOCYTES

0 HOMOGENIZED DOG GRANULOCYTES

0 HOMOGENIZED DOG GRANULOCYTES, DIALYZED

DIALYSATE, HOMOGENIZED DOG GRANULOCYTES

SALINE + LYMPH (1:1)

SALINE ELUATE + LYMPH

SALINE, BOILED

CONTROL

NORMAL DOG GRANULOCYTES, FROZEN, STORED

DOG LYMPHOCYTES, FROZEN, STORED

CONTROL

CELL FREE SALINE, AFTER 2 HR INCUBATION WITH NORMAL GRANULOCYTES

FIG 9. Dog thoracic duct lymphocytes Inhibition of DNA labelling with [³H]thymidine by granulocytes (3-hr. incubation)

obtained to permit any conclusion. It is not known at present whether this inhibitory effect of granulocytes occurs with proliferating populations of normal erythroid cells. Preliminary studies of marrow showing granulocytic hyperplasia and immaturity indicate that this inhibitor effect of mature granulocytes influences the rate of DNA synthesis of granulocytic tissue. The chemical properties of the inhibitor effect are under study.

The presence of a potent inhibitor of DNA synthesis in mature normal granulocytes would be consistent with the suggested "feedback" control system of haemopoiesis as developed by Osgood (1957, 1959). It would also help to explain many observations relative to the influence of various sera on cell growth and the occasional striking suppression of the leukaemic process by blood transfusion (Wetherley-Mein and Cottom, 1956; Bessis and Bernard, 1947). The rapidity with which the haematological changes may occur after the transfusion in such cases is not inconsistent with the leucopenic effect being one of suppression of DNA synthesis and cell division, since the non-dividing granulocytic population has a brief lifespan. The observations by Bierman and co-workers (1957) concerning the therapeutic effect of white cell products in acute lymphoblastic leukaemia are pertinent to these data. Such a mechanism would also elucidate the results of leucocytapheresis.

It has been pointed out previously that the removal of cells peripherally accelerates the release of granulocytes from the marrow reserve and promotes an increase in mitotic activity. Conversely, the intravenous infusion of large numbers of mature granulocytes in dogs inhibits the release of cells from the marrow, as shown in Fig. 12. Here the normal unlabelled cells obtained by leucocytapheresis were infused at a time when the cells with labelled DNA would normally have entered the blood from the marrow. It will be observed that the appearance of labelled cells was delayed for about 24 hours, and that the slope of the subsequent increment in new cells was similar to that in the

solution following incubation of a high concentration of granulocytes for two hours at 37°. The inhibitory effect may be a secretory product of the granulocyte. These cells were as much undamaged as is possible after this type of handling and were 90 per cent viable by the criteria of vital staining characteristics and motility.

Normal human granulocytes, intact or disintegrated, also inhibit the uptake of label into the DNA of dog lymphocytes

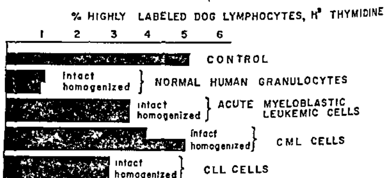


FIG. 11. The effect of human blood cells on the uptake of [³H]thymidine by dog thoracic duct lymphocytes *in vitro*. In each instance the intact or homogenized cells were added to dog lymphocytes in a 1 to 1 ratio. Incubation time with the isotope, three hours at 37°.
CML—Chronic myelogenous leukaemia
CLL—Chronic lymphocytic leukaemia

(Fig. 10). Dog lymphocytes and erythrocytes do not show much inhibition, whether intact or disintegrated. Cells from one patient with acute myelocytic leukaemia and one patient each *with chronic granulocytic leukaemia and chronic lymphocytic* leukaemia in relapse fail to inhibit DNA synthesis by dog thoracic duct lymphocytes as much as normal cells (Fig. 11). This was true with the intact cells and with disintegrated cells. The *inhibitor* effect of normal human granulocytes on leukaemic cells is a subject of present study, although sufficient data have not been

fusion of granulocytes in a manner similar to the effect of red cell transfusion on erythropoiesis It is hoped that correlation of this type of data with alterations in mitotic activity will provide information as to how this suppression of cell production comes about.

If disintegrated granulocytes are administered intravenously, a severe reaction ensues, as described by Weisberger, Heinle and Hannah (1949). In dogs this is characterized by fever, leucopenia followed by leucocytosis, intravascular clotting and defibrination, followed by a haemorrhagic state, convulsions and tachycardia. Much of the reaction is related to the release of thromboplastic and pyrogenic substances. The reaction is associated with a massive release of granulocytes from the marrow as shown in Fig. 1, and this occurs even though the cell fragments are administered via the splenic artery in an attempt to offset the systemic toxicity. This severe reaction, which is probably non-specific, prevents the demonstration of *in vivo* inhibition of granulocytopoiesis by crude products of disintegrated granulocytes. Nevertheless, the evidence for the control of granulocyte release from the marrow being geared, in some way, to the mature granulocyte is more than suggestive. It is attractive to consider this control mechanism as being related to the inhibitory effect of some product of the mature granulocyte on cell proliferation, as *shown in the foregoing experiments with immature lymphocytes.*

The preponderance of normal myeloid marrow consists of maturing cells in the marrow granulocyte reserve. If these more mature cells inhibit the growth rate of precursors, then the removal of mature cells at an accelerated rate, as in response to leucocytapheresis or infection, will increase the rate of proliferation simply by reducing the inhibitory effect of the mature cells in the environment of their precursors. This hypothesis has the merit of simplicity, though much work must be done to establish its validity. The implications of this "built-in" control of granulocyte production are obvious, and the possible relationship of

steady-state animal. If new cells continued to enter at the same rate as normal during and after the infusion, and the delay was due entirely to persistence of the infused unlabelled cells in the circulation (and hence a larger unlabelled pool to be replaced by labelled cells), the slope would be less steep than normal. It would

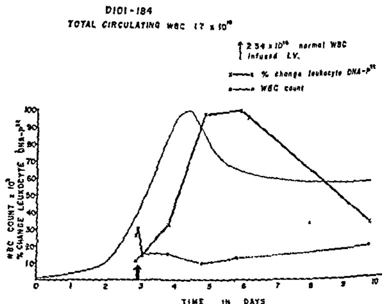


Fig 12 Delay in release of [^{32}P]DNA labelled granulocytes from the marrow into the peripheral blood in response to the intravenous infusion of normal homologous unlabelled granulocytes obtained by leucocytapheresis

appear that the entry of labelled cells from the marrow reserve was temporarily stopped by the infusion, to be resumed at the same rate after the excess cells in the periphery had been eliminated. This interpretation is predicated on the assumption that the infused cells function and survive normally—an assumption which is difficult to establish Therefore, the data suggest but do not prove that granulocytopoiesis is suppressed by hypertrans-

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[Discussion of this paper was postponed until after the short paper given by Miss M A. Maloney —Eds.]

this system to the leukaemic state should afford an area of interesting study for the future in view of the preliminary evidence that leukaemic cells are deficient in the inhibitor effect.

Summary

An attempt has been made to review some of the features of granulocyte production, distribution and utilization which seem established. The comparison of the normal state to the leukaemic situation is based on the marked alterations in these features of granulocyte physiology that occur in leukaemia. It is pointed out that whereas normal granulopoiesis is confined to the marrow and the maturation of cells occurs largely in the marrow, proliferation and maturation (if the latter occurs at all) also take place in the blood of leukaemia patients. This not only invalidates the determination of cell survival time from labelling experiments, but greatly complicates the interpretation of DNA labelling experiments in terms of the rate of cell division. Evidence is presented that the immature cell, whether normal or abnormal, remains in the circulating blood for a longer time than the mature granulocyte.

Data are presented showing great differences in the degree of DNA synthesis in the blood cells from various conditions, as measured by ^{32}P and [^{3}H]thymidine labelling *in vitro*. These results suggest that the degree of DNA synthesis is not more, and is often less, in those cell populations which appear more immature morphologically.

The finding that normal human and canine granulocytes contain a potent inhibitor of DNA synthesis by dog immature lymphocytes is described. The lack of inhibitor effect by leukaemic cells in this system is shown. These findings are believed to be consistent with the concept, expressed by Osgood, that the mature normal cell may contain an inhibitor(s) which suppresses precursor proliferation

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Short communication

RADIATION EFFECTS ON NEUTROPHIL BALANCE*

MARY A. MALONEY and HARVEY M. PATT

Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Illinois

POPULATION relationships of the neutrophil system have been examined in recent studies with the result that a somewhat clearer picture of the parameters of neutrophil balance has begun to emerge. Perhaps the most conspicuous advance in our understanding concerns the temporal and spatial distribution of neutrophils, which has profound implications for interpretation of stabilizing mechanisms. The investigations by Craddock (1959), as well as those in our laboratory (Patt, Maloney and Jackson, 1957), have established the importance of the marrow reservoir in the response to a demand situation brought about, for example, by leucocytapheresis, leucocyte antiserum, or sterile inflammation. It would appear that transfer of neutrophils from extramedullary tissue to blood is minimal under such conditions, and this receives support from theoretical considerations (Patt and Maloney, 1959) based upon recent estimates of the time course of neutrophils in blood (Mauer *et al.*, 1959; Patt and Maloney, 1959).

Radioautography with selectively incorporated radioactive precursors, e.g. tritiated thymidine, has provided an important approach to evaluation of cell population dynamics. We have observed in studies with dogs that neutrophil precursors divide every 10 to 20 hours (Patt and Maloney, 1959). After the capacity

* This work was performed under the auspices of the U S Atomic Energy Commission

for cell division is lost, the maturing cells spend about three days in the marrow before being released to the blood; their disappearance from the circulation is rapid and apparently represents a random process with a mean time of eight hours or less. Although intimate details of the normal population kinetics, particularly *of the flow of cells from one proliferating class to another*, have not yet been resolved, the approximations that have emerged provide a framework for evaluation of departures from the steady state. In this communication we wish to examine mainly the sequence of events in blood after lethal X-irradiation in the light of present understanding of neutrophil balance.

It will be recalled that the haematological effects of irradiation are generally attributable to aberrations in production Blood neutrophil changes are characterized by a transient increase during the first hours after exposure followed by a progressively developing decrease with levelling or slight rise during the second week, and a further sharp decline or eventual recovery. Experiments were performed in an attempt to answer the following questions:

(1) Is there an accelerated release of mature cells during the first hours after irradiation?
(2) Is there an initial alteration in the maturation process?
(3) What is the pattern of marrow activity as neutropenia develops?

Studies were made on beagles (one year old, 6–8 kg) with a littermate serving as a control in each instance. Total body X-radiation was given in accordance with the following factors: 250 kv, 5–6 r/minute, half-value layer (HVL) 2·18 mm. Cu. The total dose (air) was 500 r with half of the dose delivered to each lateral aspect. Tritiated thymidine (specific activity 360 mc/m-mole) was injected intravenously in a dose of 300μc/kg. body weight. Marrow samples were obtained under local anaesthesia by aspiration from the femur or iliac crest and blood samples

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marrow at this time, it should be possible to discern by frequent blood sampling whether a brief accelerated release of cells from marrow contributes to the early neutrophilia. The results, shown in Fig. 1, reveal that there is no increase in either the percentage or number of labelled neutrophils in blood of the irradiated relative to the non-irradiated dog. The curves for the irradiated and non-irradiated dogs are nearly identical during the first ten hours. The subsequent transient decrease in labelled cells in the irradiated dog coincides with the maximum neutrophilia; the ensuing pattern is similar in both dogs. The important point is that labelled cells were not increased during the neutrophilic period. It is inferred from this that the early neutrophilia is dependent upon a peripheral redistribution rather than a release of newly formed cells from marrow. It will be noted that this *effect is comparable to the adrenaline response, which is probably* representative of the mobilization of neutrophils from readily available intravascular reservoirs.

Neutrophil maturation

In this study, one of a pair of littermates was X-irradiated with 500 r. six hours after injection of tritiated thymidine. It was anticipated from our previous work (Patt and Maloney, 1959) that the majority of cells synthesizing deoxyribonucleic acid (DNA) at the time of thymidine administration would have completed mitosis prior to irradiation. Thus, most of the labelled population at the time of exposure would consist of proliferating cells in early interphase and of transitional myelocytes in early differentiation to metamyelocytes. A radiation effect on the pattern of maturation and release from marrow would be reflected several days later by the events in the blood

in circulation is reached at about the same time in both the

were taken from the external jugular vein. The procedures for radioautography and analysis were similar to those described previously (Maloney and Patt, 1958). Counts of labelled cells in marrow and blood were generally based on enumeration of 1,000 myeloid cells; in several instances of severe neutropenia, however, blood analysis was confined to 200 neutrophils.

Early neutrophilia in radiation injury

Two littermates were injected with tritiated thymidine, and of these one animal received 500 r. X-radiation three days later. At

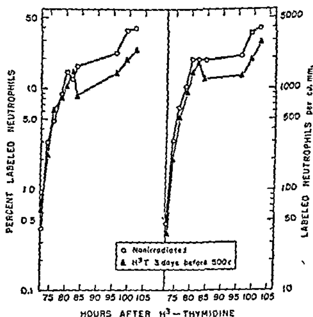


FIG. 1. Appearance of labelled neutrophils in peripheral blood of a dog irradiated three days after injection of tritiated thymidine (H^3T).

the time of irradiation less than one per cent of labelled neutrophils was detectable in the circulation. Since there is a large population of labelled cells, including segmented forms, in the

In an earlier study (Patt and Maloney, 1960), whole-body X-radiation (400 r.) was given 30 hours after injection of tritiated thymidine when there was an appreciable number of labelled metamyelocytes but no labelled band or segmented cells. In this instance, there is reason to believe that the labelled proliferating cells would be more randomly distributed in the cell cycle at the time of irradiation. Hence, the bias in the percentage of labelled differentiating cells would be minimized. This was borne out by the results, which indicated that the maturation of metamyelocyte to segmented cell proceeded in a normal fashion. From this and the foregoing, it would appear that differentiation of the progeny of the last myelocyte division and their release from marrow are not affected during the first few days after irradiation. These findings amplify our previous observation (Patt and Maloney, 1960) that the neutrophil blood level may be buffered for several days after lethal exposure by cells in the differentiating marrow pool.

Marrow activity during developing neutropenia

In this investigation, one of a pair of littermates was X-irradiated with 500 r.; three days after irradiation both dogs were given tritiated thymidine. Although the attempt at recovery is almost invariably abortive at this dose level, the three-day interval was chosen in order to study the sequence of events as the developing marrow pool becomes greatly depleted; complementary studies will be made at other intervals as well. Marrow analyses, particularly in regard to the proliferating elements, have not been compl[illegible]
tu[illegible]
lai[illegible]
may be a consequence primarily of a decrease in the time spent by the segmented forms in marrow rather than of an acceleration of neutrophil maturation. Although this requires further study, a shortened sojourn of segmented cells in marrow represents a

irradiated and control animals, accelerated transit through marrow is probably not a major contributing factor. This is supported by the fact that labelled metamyelocytes and band cells appear and reach a maximum value at the same time in both dogs. It is noteworthy that the percentage of positive cells in subsequent marrow samples is considerably greater in the irradiated animal

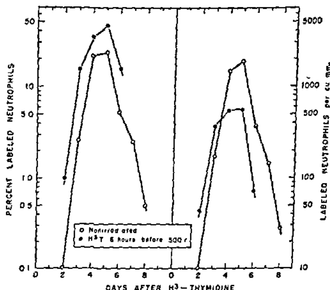


FIG 2 Appearance of labelled neutrophils in peripheral blood of a dog irradiated six hours after injection of tritiated thymidine (H^3T).

This may be an expression of preferential injury to the unlabelled cells capable of proliferation owing to the choice of the interval between labelling and irradiation. Such a distribution of radiosensitivities between the labelled and unlabelled proliferating populations would be reflected in the percentage of tagged neutrophils in blood. That labelled proliferating cells are also damaged is apparent from the decreased number of labelled neutrophils in blood relative to the control (Fig. 2).

to depletion of a succeeding unlabelled population; in the latter to preferential sterilization of a preceding unlabelled population.

The blood pattern is depicted in Fig. 3, where it will be seen that labelled neutrophils appear between the first and second days after thymidine injection in the irradiated animal and a day later in the control With this exception, the curves describing the percentage of labelled cells are remarkably similar, and we may note, in particular, that there appears to be no difference in the rate of disappearance of labelled cells from blood in the irradiated and non-irradiated animals. The number of cells produced after irradiation is, of course, considerably depressed, and this is reflected in the concentration of labelled neutrophils. That some production of cells continues, albeit at a low level, is apparent from the rate of disappearance of all neutrophils in relation to that of the labelled population. It is significant that earlier release of segmented cells from marrow coincides with depletion of neutrophils in the marrow reservoir, and that there is no indication of an altered disappearance of neutrophils from blood as a consequence of lethal irradiation.

Summary

When tritiated thymidine is administered some time in advance of irradiation, the fate of a cohort of labelled cells may be followed under circumstances where new cell formation is greatly curtailed. The results of such experiments, performed in dogs, suggest (1) that the early transient neutrophilia is not due to an accelerated release from marrow, but rather probably represents a mobilization from vascular reservoirs, and (2) that the maturation of cells derived from myelocyte mitoses and their release from marrow are essentially unaltered during the first few days after lethal irradiation.

In another study tritiated thymidine was injected three days after irradiation. In this case labelled maturing neutrophils were advancing into a depleted marrow population. The results are

reasonable initial response to a progressively developing neutropenia; the relative increase in band cells in blood as neutropenia becomes profound is a further manifestation of this response. This provides another indication of the intimate relationship between the marrow reservoir and the level of circulating

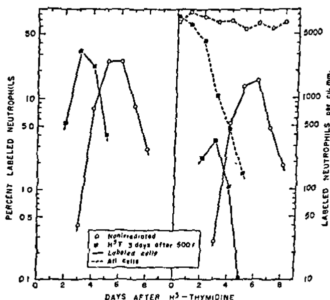


Fig. 3 Appearance of labelled neutrophils in peripheral blood of a dog irradiated three days prior to injection of tritiated thymidine (H^3T).

neutrophils (Craddock, 1959; Patt and Maloney, 1960; Patt, Maloney and Jackson, 1957). It is of interest that the percentage of labelled metamyelocytes and band cells is considerably greater than in the control and this can be explained by the severe depletion of differentiating elements in marrow four to six days after irradiation. It will be recalled that the percentage of labelled maturing cells was also increased with irradiation six hours after thymidine injection. In the former instance this can be attributed

marked acceleration in the normal rate of appearance of the ^{32}P-labelled granulocyte for a brief period of time. If we infuse normal homologous granulocytes from an unlabelled animal into an animal whose

subsequent time-course is one of a delay of about 24 hours and then

but we think this indicates that, just as when you hypertransfuse with red cells you get an inhibition of erythropoiesis, so this is a hypertransfusion with granulocytes which at least inhibits the release of granulocytes from the marrow maturation pool Whether it inhibits mitotic rate is something which we have to get more data on But this is the type of phenomenon which leads us to believe that a granulocyte may well have an inhibitory effect on its own precursors. We have not shown this, but we believe that it may be true, and if it is it makes a very concise and simple situation so far as controlling granulocyte proliferation is concerned, because the normal marrow contains a majority of its granulocytic cells in the mature and maturing form. If these cells contain an inhibitor, and if they are called into the periphery by any variety of stimulus, all one is doing is to remove a certain amount of inhibitor effect from the marrow and permit an accentuation or acceleration of mitotic rate.

Dr. Schneiberg in Warsaw who works with Jaffa sent me a manuscript about work in his laboratory in which he followed the marrow response before, during and after injection of a bacterial endotoxin in normal humans. He recorded an almost instantaneous increase in the proliferative activity in the late myeloid granulocyte precursors This is a possibility that we are very intrigued with, but we cannot give any definitive data at the moment in any cell system except the proliferating lymphocyte in a population.

Lajtha We tried to make some Ehrlich ascites cells grow and did not succeed We played around with embryo extract and then realized that in some systems you do not have to use embryo extract because white cells work just as well, so we made a white cell extract and tried

suggestive of a shortened time in marrow, due primarily to the decreased sojourn of segmented cells. In the various experiments, there was no difference between irradiated and normal dogs in the rate of loss of labelled neutrophils from blood. These findings are germane to interpretation of the sequence of events in blood consequent to irradiation.

Acknowledgments

The authors wish to express their thanks to Miss Charlotte Weber and Miss Eugenia Jackson for technical assistance.

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DISCUSSION

Gordon: Dr. Craddock, you have presented interesting evidence for the ability of viable granulocytes to inhibit DNA synthesis in lymphocytes in the *in vitro* system. Would you suppose that this mechanism could function normally as an inhibitor of lymphocyte production? A more meaningful system would involve a negative feedback of the mature granulocyte to the granulocytic precursor cells rather than to the lymphocyte progenitors.

Craddock: That is very true, and although I would like to generalize, as I implied from our material I cannot—we have not got those data.

Stohlman: If the lymphocyte is the stem cell then it is feeding back!

Craddock: If we induce leucocytapheresis, which in essence means the removal of cells from the peripheral blood, we get a transient

long time We had been wondering whether there could be an association between a prior expulsion of formed elements and the subsequent proliferation. The proliferation might be the result of taking the mature forms from the bone marrow, and in that case one ought to see it in leucocytapheresis generally. Is there any other evidence that removal of the mature cells from the blood-forming tissues sets off a proliferation of blood elements and, if so, which cell types proliferate?

Craddock: Our observations have not been detailed enough to answer that. The evidence of Schneiberg is that after the injection of an endotoxin (and endotoxin so far as we can tell, or any stimulus which causes a rapid withdrawal of cells, is pretty much the same as in leucocytapheresis) there is an instantaneous increase in proliferative activity in the myeloid granulocytic precursors.

Lamerton: This relates to something Miss Maloney said, because it could depend very much on whether the mobilization of cells is from extramedullary sites or from the bone marrow. If it is not from the bone marrow one would not expect the rapid proliferative stimulus.

Craddock: That is correct.

Yoffey: Our own not very extensive evidence helps to support your concept, Dr. Lamerton, possibly as regards the early stages of mechanical filling of empty spaces in the marrow—I know they are not empty in the sense of a vacuum, but empty in terms of cellular depletion. We had this situation on two occasions. The first was that when we gave leucocytosis-promoting factor we noted in the course of four hours a discharge of granulocytes and what seemed to us to be an uptake of lymphocytes—irrespective of the stem cell question that is a simple quantitative observation The other experiment, which we performed some years ago and are repeating much more extensively, concerned the changes following *Staphylococcus aureus* vaccine Again we got the same two changes—we called it the L·G inversion, granulocytes going out and lymphocytes going in—followed by a big increase later in granulopoiesis. We felt inclined on the whole, particularly as there was no known chemotactic substance which could influence the lymphocyte, to attribute it largely to mechanical factors Whether that is

to see whether it worked. Not only did it not work, but it very profoundly inhibited the thymidine incorporation into DNA of Ehrlich ascites cells *in vitro* as well as the incorporation of [^{14}C]formate into these cells. Formate under the incubating condition goes into DNA, RNA, protein and a number of other compounds. It was therefore a fairly non-specific depression of all activities. I think this evidence, combined with your observation that it inhibits thymidine incorporation into lymphocytes and release of mature cells from the marrow, may be an indication of a non-specific toxic effect rather than a specific feedback mechanism.

Craddock: This is entirely possible.

Lajtha: What is very interesting in your data is that this effect is not present in the same type of cells if they come from chronic granulocytic leukaemia.

Stohlman: L. Schroeder and G. Brecher at our institution are working on the *in vitro* uptake of thymidine and the preliminary results are of interest When human white cells were incubated with tritiated thymidine a certain percentage of the cells were labelled. With continued incubation the intensity of the label increased, but the percentage of labelled cells did not. At least tentatively these workers feel that cells that are in DNA synthesis at the time they are withdrawn from the body will continue to synthesize DNA, but new cells will not go into DNA synthesis. Nor have they seen any evidence for division of these cells.

Craddock. We have made similar observations, but it is not a uniform phenomenon. Sometimes certain leukaemic cells do and others do not increase the intensity of labelling over a longer incubation period, while the percentage of cells labelled remains the same.

Lamerton. We may have an example in some of our chronic irradiation work of a local inhibitory effect by mature cells in the bone marrow. We have been continuously irradiating rats at various dose rates, and at 50 rads/day there is a fall in platelets, mononuclears and polymorphocytes for about 20 days, and then there is a rise. This rise occurs at the same time in all those three elements and therefore looks more like the expulsion of formed elements from various sites than a true regeneration. But this is then followed by true proliferation and regeneration, and maintenance of the normal blood level for quite a

and Stodtmeister, R. [1955; 1956]. *Z. Zellforsch.*, 43, 195; 45, 328) published some work on the closed circulation in the bone marrow. When one depletes the bone marrow with irradiation there is a very limited amount of distension that the sinusoidal walls will tolerate. After all, the marrow space is relatively speaking of fixed volume, and if one destroys one population the only thing that can happen is dilatation with blood. The sinusoids burst and frank haemorrhage into the marrow ensues. I have often wondered if there is a simple mechanical control mechanism and not a humoral control. When one forces cells into the circulation there are tension stretch reflexes that are set up in the sinusoidal walls that are so intimately connected with the proliferating cells. Their stimulus may be actually a mechanical one locally, and we may be kidding ourselves when we talk about all these hypothetical circulating inhibitory or stimulating factors. There may be an autonomous process going on of division and it may be a matter of available space.

Yoffey: We have some data not quite ready for publication on the special features of the vasculature of the marrow. The recent studies of the living marrow circulation by P Brånemark (1959. *Scand. J. clin. Lab. Invest.*, **11**, Suppl. 38) show rather astonishingly that the living sinusoids can dilate quite rapidly, stay dilated, and that you can have groups of cells stationary on one side of the sinusoids while the main flow of blood streams past them. All sorts of things can happen to those sinusoids which we thought to be relatively inert structures. The olde[illegible] the[illegible] gro[illegible] gro[illegible] cells themselves were non-motile

Linman: Multiple daily injections of the thermostable or ether-soluble fractions of plasmas from patients with polycythaemia vera are capable of inducing granulocytosis in normal rats. The leucocytosis persists as long as the injections are continued and is accompanied by myeloid granulocytic hyperplasia. The white counts return promptly to normal after the injections are stopped I wonder if we might not be dealing with some form of an activator-inhibitor complex. Perhaps we are approaching the same problem from different directions.

was just the opposite. He had his feedback, but from the granulocytic breakdown he got nucleic acid derivatives, which stimulated fresh proliferation, even to the point of producing myeloid metaplasia and that was the basis of the sodium nucleinate therapy for agranulocytosis

Craddock: I may be muddying the waters here with a non-specific phenomenon, but we will pursue it very actively.

Lamerton: An inhibitory action of mature cells which depended on actual contact, or at least very close proximity of cells, would indicate that the control of new production which is exercised by the mature cells comes only from those which are within all blood-forming tissues and not from those in the peripheral blood or elsewhere.

Gordon: Dr. Craddock, would you agree entirely with Dr. Lajtha's suggestion that the inhibitory action exerted by granulocytes on DNA synthesis in lymphocytes is a toxic one, especially since your data indicate that normal or destroyed lymphocytes do not inhibit incorporation of the label in your *in vitro* system? If Dr. Lajtha is correct, it would mean that the granulocytes have a toxic action not exerted by lymphocytes.

Lajtha: Yes. Furthermore it is not possessed by leukaemic granulocytes. The question is: is this toxic substance connected with the alkaline phosphatase or similar materials present neither in lymphocytes nor in leukaemic granulocytes?

Craddock. There could be a variety of materials that are missing from leukaemic cells.

Stohlman: What about cells from patients with myeloid metaplasia?

Craddock: We are not certain about these cells. The only other tissue that we are certain does not affect this phenomenon is the erythroid cell. We are attempting to study the toxic effect of granulocytes on lymphocytes with the co-operation of someone at our institution who is interested in time-lapse phase microscopy of cells. We have not been able to show any apparent damaging effect of this phenomenon on motility and other morphological properties of the lymphocytes exposed to this inhibitory effect from intact or destroyed granulocytes, or apparently from the secretory products of intact granulocytes; the cells remain viable as far as we can tell, and there is no evidence of destruction of the cells by this principle, whatever it is.

Cronkite: Fliedner and co-workers (Fliedner, T. M., Sandkuhler, S.,

myeloid leukaemia, can be shown to cause an increase of the osmotic fragility. If this is a toxic action, possibly you can find an increase of osmotic fragility.

Stohlman: Dr. Craddock, do you consider the evidence for a long lymphocyte lifespan is inadequate? To me the only possible interpretation for the data of Hamilton and Ottesen, as well as that presented by Cronkite today, is that the lymphocyte is long-lived.

Craddock: I think their data are open to the same criticism as the type of data I showed in the leukaemic individual, as regards telling anything about the length of survival of the final product of proliferation.

Hulse: I would like to support Miss Maloney when she says that there is no accelerated maturation of the granulocyte series after irradiation.

Maloney: I cannot offer any information about the question of abnormal myelocytes in marrow after irradiation. For the moment, we have focused our attention on the non-proliferative, differentiating myelocytic population. There is no evidence of an acceleration in maturation for this population. That is from metamyelocyte to segmented neutrophil.

Hulse: One certainly does get a segmented myelocyte after irradiation, and many people in the past have suggested this is due to an accelerated maturation. You did not find any evidence of accelerated maturation and I would like to support your findings by saying that I do not think that the presence of hypersegmented neutrophils and segmented myelocytes and promyelocytes indicates an accelerated maturation The segmentation of myelocytes and promyelocytes is, I think, merely a change in the nucleus. The cytoplasm is not changed at all, it still has azurophilic granules, etc One also gets a hypersegmentation of the nucleus in adult neutrophils. It looks as if there has been some damage to the nucleus of these cells, but they can still mature normally, or at least their cytoplasm can. The nucleus of a myelocyte becomes segmented after irradiation and eventually this may become a hypersegmented neutrophil.

Yoffey: I have been wondering when this business of the asynchronism, which I think Di Guglielmo first emphasized, would come up. We have seen some good examples in our marrow radiation recoveries. We were often perplexed whether to call a cell a large metamyelocyte

Craddock: It seems quite reasonable that there would be both activators and inhibitors. I hesitate to stress my own data because of the doubt I have as to whether it is a specific phenomenon. Whether it is a specific inhibitor or [illegible]

[illegible]: I would not subscribe completely to Dr. Cronkite's statement that humoral factors are not operating in the release and production of leucocytes. I will present evidence on Thursday that such factors do indeed exist.

With respect to the endotoxin effects described by Dr. Craddock, Professor Komiya and his associates in Tokyo have demonstrated the appearance of leucocytosis-inducing factors in the plasma of animals injected with vaccines. They have sent us a sample of their "neutropoietin" evoked in the plasma of rabbits by typhoid vaccine. We have found that a single injection of 250 µg. of this material, administered intravenously into rats, resulted in significant peripheral neutrophilia, in the course of four hours, which is a shorter time interval than that necessary for the peripheral leucocytosis that follows the injection of typhoid vaccine itself. More recently, in confirmation of Komiya's results, we have found that leucocytosis-inducing activity also appears in the plasma of 250 g. rats injected with 0·5 ml. of typhoid-paratyphoid vaccine (New York City Health Department). These experiments support our growing feeling that there are circulating factors which control the rate of release and possibly the production of leucocytes.

Cronkite: I was not implying that there was nothing which would control the release. I was thinking more of the proliferation at a more immature stage. In addition to the Japanese work a large amount of work was done by R. S. Farr and colleagues on the problem of the pyrogenic response, the plasma factors that are produced and activated that influence fever and leucocytosis.

Astaldi: Dr. Craddock, you say that with your granulocytic material you have not been able to show morphological separations in lymphocytes by phase microscopy or anything else. I would like to suggest that you should also look at the osmotic fragility. Some cytotoxic substances when tested *in vitro* with leucocytes or lymphocytes, and also *in vivo* during treatment of patients with lymphatic leukaemia or

tion of the nucleus and the maturation of the cytoplasm. Similarly there would be a discrepancy between the apparent maturity of the nucleus and the glycogen content of the cytoplasm, such as Prof. Astaldi found. It seems to me that a cell in the myeloid series normally has a nucleus which is inclined to undergo segmentation. It undergoes segmentation as it gets older, so perhaps segmentation of the myeloid cells is a form of nuclear degeneration. When we irradiate perhaps we are causing nuclear degeneration to set in earlier, and therefore we see earlier segmentation and eventually hypersegmentation in this particular group of cells.

Astaldi: Is this without maturation of the cytoplasm?

Hulse: Only at the normal rate. The nuclear changes have been a red herring—they have led people astray. The changes are really a form of nuclear degeneration.

Astaldi: Do the structures contained in the cytoplasm not mature so well?

Hulse: They may mature, only they are out of line with the apparent maturation of the nucleus. When a promyelocyte with a segmented nucleus matures its nucleus, having become segmented much earlier in development than usual, will soon look like that of an adult neutrophil. We may infer from Miss Maloney's results that its cytoplasm will mature at a normal rate and will therefore be at a much earlier stage of development. Hence the discrepancy between nuclear and cytoplasmic maturation. The eventual outcome of such changes may be seen 24 to 48 hours after a high dose of radiation when neutrophils are present with nuclei so segmented that they look like a bunch of grapes.

Lajtha: You may certainly have cytoplasmic processes which are not affected by irradiation, such as iron uptake in normoblasts. Normally a basophilic normoblast would increase its iron content from X to X+ and when it divides the two daughter cells will have half that [illegible]

We have seen that with ^{59}Fe in cultures. Although the polychromatic normoblasts would normally have a mean grain count of, say, 25, after a certain dose of radiation we did get a number of polychromatic normoblasts with higher grain counts. We also saw this in the

when the nucleus was well past the myelocyte stage, and was markedly indented or even segmented, but no granules had as yet appeared, or whether still to call it a myelocyte.

Hulse: In your department the dose was something like 150 r. but if you go up to, say, several thousand röntgens then you get a much larger number of myelocytes and promyelocytes with segmented nuclei.

Astaldi: The maturation time of the cells after their irradiation probably is not prolonged. I should like to suggest that irradiation may disturb the process of maturation in such a way that asynchronism may result, as Prof. Yoffey pointed out. For instance during maturation of white cells, both lymphocytes and granulocytes, there is a progressive increase in the glycogen content; that is to say, the cellular glycogen increases when passing from lymphoblast to prolymphocyte and small lymphocyte, as well as when passing from myeloblast to promyelocyte, myelocyte, metamyelocyte and granulocyte. If you irradiate these cells, you may observe that the glycogen content does not increase normally, so that these cells mature with a defect in their glycogen content. This is one of the examples of the paraplasm not maturing completely after irradiation and it may be connected with disturbances in the enzymes, or something else which interferes with the glycogen synthesis in leucocytes.

Fichtelius: Weicker in Bonn states that there is a reduction mitosis at the myelocyte stage. In pernicious anaemia this reduction mitosis is inhibited, and as a consequence of this the large metamyelocytes and hypersegmented neutrophils develop. Weicker counted the number of drumsticks in females with pernicious anaemia and found that the number was twice that in normal females This was considered a proof of his theory. Are these conditions considered in the case of your hypersegmented neutrophils, Dr. Hulse?

Hulse: I don't know whether the hypersegmented polymorph in pernicious anaemia is the same sort of thing as the hypersegmented polymorph after irradiation. I would rather think it was not. If a myelocyte is irradiated then its nucleus may become segmented as a consequence of the irradiation. The cell then, from what Miss Maloney says, may still mature at the same rate but I think that it may segment further as it matures This leads to a discrepancy between the matura-

ferentiate, and appear in the peripheral blood. We have not completed analysis of the proliferative elements for generation time and mitotic time of the irradiated animal relative to the non-irradiated animal.

Craddock: Miss Maloney, after 500 r. and at the time their labelled cells were declining, were the dogs very sick?

Maloney: Yes.

Craddock: And yet the fall was pretty similar to that in the non-irradiated animals We observed similar findings with irradiated animals using the DNA-^{32}P labelling technique and were unable to interpret these, since we felt that there should have been a much more rapid decline in the irradiated sick animal, where obviously many things were going on. I think your beautiful data would suggest that our contention that the normal mature polymorphocyte is a very short-lived cell, at least so far as its stay in the peripheral blood is concerned, is indeed correct.

myelocytic series using ^{35}S which is not taken up by the band cells at all It takes about 24 to 48 hours for the myelocytes to come to the band cell, and usually the band cells have half the grain counts originally present in the myelocytes. If you irradiate or give colchicine to such cultures, you will get band cells with twice the normal grain count. The number is not very large admittedly—many of these cells will probably die in mitosis. Nevertheless these data indicate that there is one aspect of cytoplasmic differentiation which is not inhibited, and that maturation, at least in respect of sulphate or iron uptake, can go on. Other processes, such as glycogen synthesis which Dr. Astaldi mentioned, may be affected.

Cronkite: In the HeLa cell tissue culture R. B. Painter and J. S. Robertson (1959. *Radiat. Res.*, **11**, 206) have shown that irradiation does not eradicate the capacity to synthesize DNA. It continues, possibly at a slower rate. The ability to stop doubling the amount of DNA is apparently lost and cells continue to incorporate labelling material. Comparable observations have been made by Fliedner in irradiated rats. They continue to synthesize DNA apparently for longer periods of time and in more than the tetraploid amount, yet still have the capacity to divide but produce bizarre mitotic figures and cells A comparable sequence was observed in the Oak Ridge casualties using *in vitro* uptake. A general interpretation that I would like to propose is that these cells which have a greater than tetraploid amount of DNA are unable to divide at times, but they continue to mature and when they start segmenting they have a lot more DNA to segment. Large amounts of labelling appear in these cells, completely out of any of the experience in ordinary non-irradiated cells. This is seen *in vitro* and *in vivo*.

Jacobson. In the first 24 hours after irradiation of the rabbit with 800 r. there are two waves in the peripheral granulocytes Have you any information on the mechanism of the two waves, Miss Maloney? And have you any idea about the puzzling problem of what the abortive rise is?

Maloney: We did not observe a second neutrophilia in this animal. I have no information concerning the origin of cells whose progeny contribute to the abortive rise in the peripheral leucocytes. However, these cells do incorporate tritiated thymidine, and they divide, dif-

ferentiate, and appear in the peripheral blood. We have not completed analysis of the proliferative elements for generation time and mitotic time of the irradiated animal relative to the non-irradiated animal.

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Short communication

THE MOBILIZATION OF VITAMIN B_{12} IN RESPONSE TO ACUTE BLOOD LOSS

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PRELIMINARY observations on a factor possibly involved in the stimulation or control of haemopoiesis will be reported here. These observations were made in rats in the course of studies on the dynamics of vitamin B_{12} distribution during increased blood production in response to acute blood loss.

Vitamin $[^{60}Co]B_{12}$ when administered parenterally to rats is concentrated in several organs, especially in intestines, kidney and liver. Twenty-four hours after the injection the radioactivity begins to decrease in the intestines, kidney and other organs. Only in the liver is there a continuous rise in radioactivity lasting up to seven days after the injection. The physiological factors governing the release of B_{12} from its stores are unknown. It is to be expected that increased demands such as increased blood regeneration would be associated with release of B_{12} from its stores. With the purpose of investigating this assumption, acute anaemia was produced in some rats by bleeding, and in others by haemolysis, and the effect of these procedures on the release of B_{12} from its storage compartments was studied.

The animals which were bled and examined 24 hours later showed a significant loss of labelled B_{12} from kidney and liver. In rats in which the anaemia was produced by haemolysis no difference in the total radioactivity of the kidneys and liver was

observed in comparison with the control rats, although the degree of anaemia was of the same magnitude in both groups. The loss of radioactivity from liver and kidney is more evident when the B_{12} lost is expressed in relation to the entire radioactivity of the animal at the time of the experiment. When the lost blood is replaced by the equivalent amount of whole blood or red cell suspension, mobilization of B_{12} from kidney and liver is prevented. On the other hand, when the withdrawn blood is replaced by the same amount of plasma, loss of radioactivity of the kidney and liver was observed equivalent to that occurring after bleeding alone. Mobilization began as soon as half an hour after the bleeding, with the maximum effect occurring in the first four hours following the haemorrhage.

It was then necessary to determine the fate of the B_{12} mobilized from its stores. It was known that only a small part of the mobilized B_{12} was excreted in urine and faeces It was therefore apparent that the released B_{12} must go into another body compartment. To test this the following experiment was carried out. The rats were treated as before, but the carcass was now subdivided into two main fractions: one consisting of the bone and muscle and the second consisting of all the remaining viscera and skin excluding liver, kidney and spleen. At the same time a sample of muscle was taken for counting, from which the total muscle radioactivity in the muscle-bone fraction could be determined. The radioactivity of the bones was also determined after they were separated from the alkaline homogenate.

It was assumed that any radioactivity not accounted for in muscle and bone would be present in the bone marrow. It was found that there was a marked fall in the radioactivity found in the viscera, with a corresponding but somewhat slower rise in the muscle-bone and bone marrow compartment. This change was present in the "bone marrow" fraction since the radioactivity of the muscle and bone remained more or less constant over the period of the experiment.

The liver and the kidney also showed significant decreases in radioactive B_{12}, although the magnitude of the phenomenon was somewhat less than in the previous experiments. This difference might have been due to the fact that somewhat older rats were used in the last experiment.

Several further observations can be made. The B_{12} radioactivity in the "bone marrow" fraction is negligible in the control animals. Under the stimulus of bleeding it rises rapidly in the first hour, then more slowly to reach the peak at 12 hours, following which there is a decline in radioactivity in 24 hours.

Corresponding with the rapid drop in the B_{12} storage compartments there is a significant rise in the first half-hour in the blood B_{12} radioactivity, which then falls rapidly to control levels.

It is clear, then, that bleeding causes mobilization of B_{12} *from* viscera, kidney and liver. This B_{12} is delivered into the blood and then passes into a portion of the body which is most probably the bone marrow.

Discussion

These experiments show that withdrawal of blood results in a remarkable shift of $[^{60}Co]B_{12}$ from its storage compartments. The loss of the comparatively small amounts of radioactive B_{12} contained in the shed blood cannot account for the considerable quantities of the vitamin mobilized from its compartment stores The lack of correlation between the quantities lost to the body and the amount of B_{12} released from the stores is also borne out by the observation that reinfusion of plasma, containing some 50 per cent of the vitamin lost, does not change the effect of bleeding on the release of B_{12}.

[illegible]

when the lost red cells are replaced by reinfused cells or by whole blood. The fact that the stimulus is absent when the acute anaemia

is produced by haemolysis is also in agreement with this conclusion. It appears that the mobilization of stored B_{12} precedes the increased blood production following the anaemic anoxia. It could be speculated that the B_{12} entering the bone marrow is in some way connected with or forms part of the primary stimulus to increased blood production as a result of acute blood loss.

The rôle of the phenomenon described in the maintenance of a constant red cell level, as a homeostatic mechanism or a feedback system, cannot at present be evaluated, and further studies are needed to throw light on this problem.

DISCUSSION

Stohlman: This difference between haemolytic anaemia and blood loss is intriguing. How rapidly is the anaemia produced in the haemolytic syndrome as compared with the blood loss, and how severe was it in the two instances? Have you tried to reinfuse damaged or frozen red cells?

Rachmilewitz: The degree of anaemia in haemolytic anaemia was certainly not less than in the bleeding anaemia. We have two sets of experiments with phenylhydrazine; in one we examined the organs 24 hours after the intravenous administration of phenylhydrazine, and in another set we examined the organs four days after, and there was no difference in effect.

Cronkite: A counterpart of this type of potential "feedback mechanism" came out of studies with platelets. If one hypertransfuses viable platelets that will circulate in the rat to a platelet level of twice to thrice the normal amount, and then lets the platelet count spontaneously decrease, it keeps going down, to perhaps 20 per cent of the pre-transfusion level, and it takes four to five days to come back up. However, if one lyophilizes the platelets, or does anything to them so that they will not circulate, such as storing them for 24 hours, there is no inhibition or secondary fall of platelet level—this remains constant. This factor that apparently temporarily turns off the production of new platelets and disturbs the steady-state equilibrium is extremely labile and its nature is not characterized.

Lamerton: Presumably the four-day inhibition would be the time taken from the proliferation of the megakaryoblast up to the production of platelets?

Cronkite: That is what I thought, but as one looks at the marrow and the spleen of the rat, the megakaryocytes look as if they ought to be making platelets, but apparently they are not.

Yoffey: You have no views on the suggestions about extramedullary sites of platelet formation?

Cronkite: No, except in rodents where it obviously goes on in the spleen, and some pathologists continue to comment on megakaryocytes in the lungs.

Lajtha: Dr. Rachmilewitz, you think that the stimulus for vitamin B_{12} release, or rather the inhibition of B_{12} release, is due to a breakdown product of the red cell, and if you decrease the normal feedback of these breakdown compounds to the liver by taking out a large proportion (in your experiments half the blood volume of the animal) then would vitamin B_{12} be released?

Rachmilewitz: It is not only liver. We have seen the storage compartments from which the B_{12} comes, and the most important is in the viscera—probably the intestines and to a lesser degree from the liver and kidney. I think there are two aspects to be considered: one is the mobilization or release of B_{12} from its stores, and the second is its entrance into the bone marrow immediately after that. Chronologically the first event is a release of B_{12}, as I have shown, and the second phase is the entering into the bone marrow, for a very short time, because in the control animals the bone marrow has almost no radioactivity. You have seen how quickly the radioactivity declines in the bone marrow.

Lajtha. What do you think this B_{12} does in the marrow? Does it stimulate nucleic acid synthesis, or synthesis of haem? If it works on nucleic acid metabolism mainly, then whether you produce anaemia by haemolysis, anoxia or bleeding, you should get the same transfer. If, however, the compound only works on haem synthesis, then you should be able to "dilute" the B_{12} transfer with haem breakdown products—as is suggested by your results with phenylh[illegible]

Rachmil[illegible]

draws in considerable quantities of B_{12}. Eight days after partial hepatectomy the regenerating liver contains almost the same amount of B_{12} as before hepatectomy, so it is probably some manifestation of regeneration of any organ, not necessarily of bone marrow.

Gordon: Partial hepatectomy entails the removal of a considerable part of the blood volume Might not this superimpose a factor of hypoxia in your hepatectomy experiments?

Rachmilewitz: This may be true, but in the regenerating liver there is a considerable rise in B_{12}, while in the case of blood loss the liver B_{12} is diminished.

Yoffey: To what extent does free haemoglobin in the blood stream have any kind of rôle as an oxygen carrier compared with the haemoglobin when it is in the red cell?

Stohlman: Are you speaking of haemolytic anaemia? I doubt if there is any free haemoglobin in the blood

Braunsteiner: Haemoglobin in the serum is normally bound to haptoglobin.

Gordon: The coelomic fluids of many invertebrates, especially the [illegible] gen [illegible] the [illegible] mal pressures of the gas. This probably constitutes an adaptive mechanism of aquatic and semi-terrestrial organisms to their environment.

Craddock: Is there any possibility that the red cell stroma, or something in the red cell, could contain a minute amount of B_{12} which could be re-utilized, and therefore the depletion of B_{12} would be lessened in the two situations?

Rachmilewitz: Whipple (Woods, W D, Hawkins, W. B., and Whipple, G H. [1958] *J. exp Med*, 108, 1) showed a few years ago that after haemolysis and bleeding B_{12} is incorporated into the stroma, but I think the quantities involved are very small. We are dealing here with the mobilization of much larger quantities than the amounts lost in the shed red cells.

Stohlman: You were measuring mobilization of cobalt, not of B_{12}—is all of the cobalt still in B_{12}?

Rachmilewitz: We now intend to do the same thing with microbiological studies to know whether the same effect will be obtained.

But it is very suggestive that cobalt-60 is present in the form of vitamin B_{12}.

Cronkite: We did a series of studies in conjunction with the Merck Corporation, trying to ascertain whether cobalt-60 for prolonged periods of time still represents B_{12}. I think the answer is that 95 per cent or more of it does for long periods of time (Rosenblum, C., and Cronkite, E. P., to be published).

Rachmilewitz: Yes, the total amount is the same.

Cronkite: We tried to do comparable studies in irradiated animals during regeneration, and we missed it.

Linman: Have you had a chance to study the effect on vitamin B_{12} of hypoxic hypoxia or exposure to lowered barometric pressures?

Rachmilewitz: Not yet, but it is on our programme.

STUDIES ON THE KINETICS OF ERYTHROPOIESIS: A MODEL OF THE ERYTHRON

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"*What's the use of their having names,*" *the Gnat said,* "*if they won't answer to them?*"

(Through the Looking-glass)

In recent years, particularly with the aid of radioactive isotopes, a great deal of information has accumulated on various aspects of the kinetics of erythropoiesis. In science, if and when sufficient data are available, it is permissible to attempt to construct a model for further investigational purposes, to be confirmed, modified, or discarded. Such an attempt is presented here, correlating available information on the nucleated red cells in the bone marrow to give a mental picture of their functioning as a unit, the erythron.

The model as described here refers to man, as most of the data presented were obtained with human bone marrow cells. First the experimental evidence will be presented, followed by its synthesis into a model, and in conclusion some hypotheses.

Experimental evidence

Most of the evidence to be cited has been reviewed previously (Lajtha, 1957). No technical details will be given here; reference is made to original sources

(1) *Rate of* ^{59}*Fe uptake* by nucleated red cells at different stages of maturation as determined on radioautographs (Lajtha and Suit,

1955; Suit *et al.*, 1957). The rate is highest in pronormoblasts, somewhat less in basophilic normoblasts, and it further decreases in the following order: early polychromatic normoblasts, late polychromatic normoblasts, marrow reticulocytes, blood reticulocytes (Fig. 1). The findings are similar with ribonucleic acid (RNA) or protein label (Lajtha, 1957).

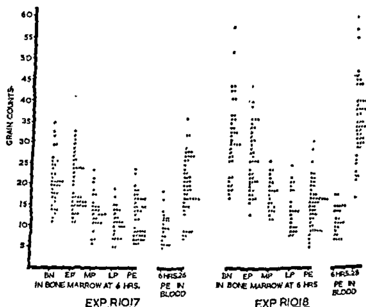


FIG 1 Spread of grain counts over rabbit normoblasts and erythrocytes after an injection of ^{59}Fe.
BN= basophilic normoblasts, EP, MP, LP= early-, middle-, and late-polychromatic normoblasts, PE= polychromatic erythrocytes (reticulocytes)
(Reproduced from Lajtha and Suit, 1955 by permission of the Editor of British Journal of Haematology)

(2) *Haemoglobin content* of normoblasts at different stages of maturation measured with u.v. absorption at 4,047 Å (Carvalho, 1954). This increases from an approximate 16 µµg. per cell in the basophilic normoblasts to just over 30 µµg. in the erythrocyte.

As the haemoglobin content of the cell increases, its RNA content (cytoplasmic basophilia) decreases.*

(3) *Appearance in blood of reticulocytes* with unexpectedly high ^{59}Fe content (Suit *et al.*, 1957). While six hours after administration of ^{59}Fe to rabbits the radioautographic grain counts over blood reticulocytes are low, by 24 hours cells appear with grain counts similar to early normoblasts (Fig. 1). As after six hours there is insufficient ^{59}Fe in the plasma to give a radioautograph, these cells could only have originated from *early normoblasts maturing without intervening cell division* (as every division would have halved the grain count).

(4) *Changing rate of the synthesis* during interphase. If the rate of synthesis of a compound is constant in a cell type (e.g. basophilic normoblasts) then the grain counts on radioautographs would show a Poisson distribution. However, grain count distribution studies indicated that the rates of synthesis of most compounds (Hb, RNA, DNA [deoxyribonucleic acid]) are changing, and are compatible with an exponentially changing rate, i.e. *increasing from "2n rate" (after mitosis) to "4n rate"* (immediately prior to mitosis), as shown in Fig. 2.

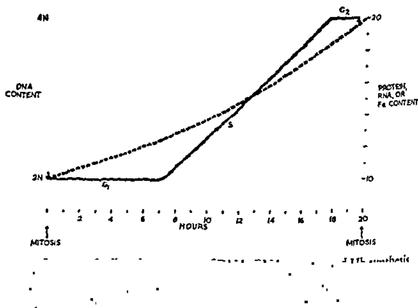
(5) *Cell cycle*. The cell cycle in respect of DNA synthesis and mitosis has been established in human bone marrow cells *in vitro* (Lajtha, Oliver and Ellis, 1954) and for the pronormoblasts and basophilic normoblasts an intermitotic cycle time of about 20 hours was found. The cycle time is somewhat longer in the polychromatic normoblasts, and there is no DNA synthesis in the late normoblasts, indicating that the latter are not dividing cells (Lajtha, 1959).

Similar cycle time for pronormoblasts has been found in dogs *in vivo*, using the ^{59}Fe grain count halving time, perhaps the best

* Note that there is a species difference in respect of (1) and (2) between man, dog and rabbit on one hand, and the rat on the other hand. In the latter the basophilic normoblasts contain no appreciable amounts of haemoglobin, and their ^{59}Fe uptake is similar to the late normoblasts (Thorell, 1947, Austoni, 1954).

direct measure of cell generation time (Alpen and Cranmore, 1959).

(6) *Lack of direct effect of erythropoietin* on normoblasts *in vitro*. Urinary erythropoietin, which exhibited marked stimulating effect on erythrocyte production in rats *in vivo*, failed to show any effect *in vitro* on the cell cycle of human normoblasts, neither did it stimulate ^{59}Fe uptake by the cells (? 10 per cent increase) It was



concluded that erythropoietin does not act by shortening the intermitotic time of the normoblasts (Alpen, Lajtha and Van Dyke, 1959).

(7) *Dilution of a labelled normoblast population* by unlabelled cells of unknown origin. Alpen and Cranmore (1959) found that while in normal dogs about 95 per cent of the pronormoblasts remain labelled for about 24 hours after ^{59}Fe labelling, in their bled dogs (stimulated erythropoiesis) the proportion of labelled cells drops to less than 20 per cent by 24 hours. As the grain count

halving time did not differ in the control and bled animals, they concluded that the unlabelled cells originate from an undifferentiated (no iron uptake) "stem" cell population. The normal "feed" from the stem population into the erythron is indicated by the fall of "per cent labelled cells" in the control dogs from 95 per cent (at 24 hours) to about 20 per cent (at 72 hours). This "feed" is accelerated by erythropoietin as shown in their bled dogs.

Similar observations were noted in this laboratory by Suit (1956) in rabbits. The control rabbits showed a high level of ^{59}Fe-labelled early normoblasts for about 24 hours, but this proportion decreased to about 70 per cent by 24 hours if the rabbits received 200 r. whole-body irradiation prior to ^{59}Fe administration (Fig. 3). As such a dose of radiation would inhibit mitosis for about 12 to 15 hours, the unlabelled cells "diluting" the labelled population (especially in view of the findings of Alpen and Cranmore, 1959) indicate feed from an undifferentiated stem population. These observations furthermore indicate that stem cells can differentiate into the erythron without intervening division

(8) *Additional information used:*

(*a*) The normal bone marrow differential count in man (i.e. ratio of pronormoblasts: basophilic normoblast: early polychromatic: late polychromatic normoblasts: reticulocytes)

(*b*) Appearance rate of a tracer dose of ^{59}Fe in the peripheral erythrocytes (normal and stimulated) in man.

(*c*) Concept of "ineffective erythropoiesis" (Finch *et al.*, 1956).

(*d*) Bone marrow erythroid cellularity (Suit, 1957).

(9) *Assumptions made:*

(*a*) It has been suggested (Alpen and Cranmore, 1959; Lajtha, 1959) that the pronormoblasts are partially self-maintaining, supplemented by differentiation of stem cells This would, however, imply a "choice" to be made by cells between dividing only or dividing and differentiating For the present model it is

taken that each cell can only produce its own kind by division, and that the stem cells are the sole source of normoblasts (notwithstanding differentiating divisions of normoblasts).

(*b*) It is assumed that the stem cell population has its own internal homeostatic regulation, and that differentiation of stem

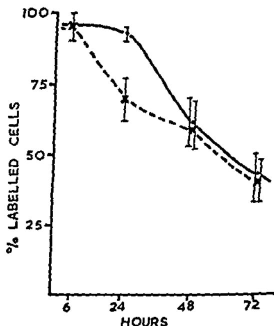


FIG 3. Changes in proportion of labelled early basophilic normoblasts in rabbits after a single dose of ^{59}Fe

Solid line = control animals, Interrupted line = 200 r. whole body irradiation immediately prior to ^{59}Fe injection

cells into the erythron is a random process, initiated by the humoral factor erythropoietin (possibly by enzyme induction in the stem cells)—a certain level of erythropoietin removing a certain *percentage* of the stem cells into the erythron.

(*c*) It is assumed that the cytoplasmic haemoglobin concentration influences nuclear functions in the normoblasts: having reached a certain concentration the cell may become incapable of further mitosis; at a later concentration the loss of nucleus may occur (? karyolysis, ? karyorrhexis, ? extrusion).

(*d*) Although not a prerequisite for the model, it is assumed that the "pronormoblast" is merely a $4n$ form of the earliest normoblasts, consequently "pronormoblasts" would be called "basophilic normoblasts" in their $2n$ state (i.e. after division).

Description of the model

Based on the foregoing, the model, as shown in Fig. 4, has been constructed. The description of events, based on this model, is as follows:

One stem cell differentiates into the erythron and takes the morphological appearance of pronormoblast. Within about 20 hours this cell will divide, but before division it may already have started iron uptake. The morphological character, however, may develop earlier than the ability to take up iron.

After division the two daughter cells, being smaller, may be called basophilic normoblasts (Ia) These cells synthesize haem at a fast rate, and, after a post-mitotic gap (G_1 period), will synthesize DNA (S period), followed by a pre-mitotic gap (G_2 period) (Fig. 2). By the time they are about halfway through their S period they are larger and may be called "pronormoblasts". After division the same cycle repeats itself (Ib). By the following cycle (Ic) the haemoglobin content of the cells has increased sufficiently to interfere with the "nuclear structure", so that the cells at the end of Ic stage will not assume the morphological appearance of "pronormoblasts".

Perhaps already in stage Ic some loss of RNA occurs from the cells (thus decreasing overall cell size and altering the morphological structure of the nucleus)—but this loss becomes obvious

in stage II when the cells assume the morphological appearance of "early polychromatic normoblasts".*

Concomitant with loss of RNA, all cell functions (iron uptake, protein synthesis, cell cycle) slow down slightly, and the intermitotic cycle of stage II cells has been taken to be 30 hours as opposed to the 20 hours of stage I cells.

The number of mitoses a cell undergoes, and the intermitotic cycle time (or "lifespan" in the case of non-dividing cells) determines the proportional distribution of cells in the erythron. As can be read from the model the distribution is

7×5	i.e. 35	("hours' worth")	pronormoblasts
6×15 } 8×20 }	„ 250	„	basophilic normoblasts
12×30	„ 360	„	early polychromatic normoblasts
16×50 } 4×18 }	„ 872	„	middle and late polychromatic normoblasts, and orthochromatic normoblasts
16×40	„ 640	„	marrow reticulocytes (if lifespan in marrow only 40 hours)

and this agrees well with the differential count in normal marrows

As the distribution of basophilic normoblasts in stages Ia, b, and c is 2·4:8, most measurements on "basophilic normoblasts" represent a predominantly Ic population (i e two divisions

* [illegible] basophilic to [illegible] In reality—as [illegible] be classified [illegible] ular division (depending on the nomenclature used) This, however, does not significantly affect the model.

"INEFFECTIVE ERYTHROPOIESIS"

Ba Pro Ba Pro Ba Ba Ba Pro Ba Ba

II'(EPo) III' IV'(ONo)
0·35 0 25-0 15
>27 012-010 >30

S--- Pro

Ba Pro Ba Pro Ba Ba Ba Pro Ba Ba

STAGE	1a 20hrs	1b 20hrs	1c 20hrs	II (EPo) 30hrs	III (MPo-LPo) 50hrs		IV (MR) 40hrs			
μμg Hb. Rate/2n cell/hr	0 5	0 5	0 5	0 33	0 25	0 2	0 17	0 15	0 12	0 10
μμg Hb. Content /cell	14 4	7 2 21 6	10 8 25 2	12 6 27 0	13 5	24 5				30

FIG 4 Kinetic model of the erythron.

S=stem cell compartment; Pro=pronormoblasts, Ba=basophilic normoblasts; EPo, MPo and LPo= early-, middle-, and late polychromatic normoblasts; MR=marrow reticulocytes, ONo=orthochromatic normoblasts Horizontal lines indicate length of intermitotic cycle or duration of stage in hours Rate of haemoglobin synthesis and haemoglobin content are expressed in μμg

removed from reticulocytes), while for similar reasons those on "pronormoblasts" represent a predominantly Ib population (i.e. three divisions removed from reticulocytes).

According to the model one stem cell differentiating into the erythron will produce 20 reticulocytes, and one stem/hour will produce 20 reticulocytes/hour, with 1,517 normoblasts in between. The daily reticulocyte production in man is about 10^{11} per day, i.e. 4×10^{9} per hour, corresponding to 5×10^{9} stem cells per day, i.e. 2×10^{8} per hour. For each stem cell entering the erythron there are 1,517 normoblasts in the erythron, therefore the number of nucleated red cells in the marrow (man) is $2\times10^{8}\times1{,}517$ i.e. about 3×10^{11}, corresponding well to the figure found by Suit (1957).

All stage II cells are preparing for mitosis, and, usually, after a 30-hour-cycle they divide, giving rise to stage III cells—these latter, because of further loss of RNA, synthesize haemoglobin at a slower rate, and as at a certain concentration of haemoglobin (> 13·5 μμg./2*n* cytoplasm) the process of "nuclear pyknosis" starts, they will not synthesize DNA and they will be incapable of further division. Cytoplasmic processes, however, continue, and after a certain haemoglobin level has been reached the nucleus is extruded or lysed and the cells become marrow reticulocytes (stage IV).*

Although all stage II cells are preparing for mitosis, it is postulated that an intricate balance exists between cytoplasmic differentiation (? haemoglobin content) and ability to divide. Thus a proportion of stage II cells (stage II′), which perhaps synthesize haemoglobin somewhat faster than the average, may reach the critical concentration of haemoglobin which initiates "nuclear pyknosis" (> 27μμg./4*n* cytoplasm) by the end of their 30 hours' cycle, and consequently they will be unable to divide. Such cells

* Stage III cells start their existence with a pure diploid nucleus. It is conceivable that this nucleus is extruded as such, but a gradual loss of nuclear material (especially RNA) is likely to occur.

will enter a different pathway as they start stage III′ with a tetraploid nucleus and twice the haemoglobin content of ordinary stage III cells. It may be more difficult to get rid of a tetraploid nucleus than of a diploid nucleus, and therefore, although (based on their iron content) these cells enter very shortly into stage IV′, they still retain their nuclei—thus instead of becoming marrow reticulocytes they become "orthochromatic" normoblasts. Such cells will lose their nuclei at such a late stage of cytoplasmic differentiation (haemoglobin content) that some may not produce reticulocytes. It is assumed in the model that this pathway also produces normal size erythrocytes, i.e. the absolute rate of haemoglobin synthesis falls steeply. However, in reality it is likely that the rate of fall is less steep than illustrated, and this would then result in slightly larger erythrocytes than normal (macrocytes).

It is expected that some unbalance of the nucleo-cytoplasmic functions may cause cell death at early stages—this would result in destruction of cells capable of iron uptake—signified on the scheme as "ineffective erythropoiesis".

Nucleic acid and protein synthesis

DNA synthesis only occurs during a limited period in interphase (S period in Fig 2) and in bone marrow cells it is situated more in the second than in the first half of interphase. The duration of S period is about ten hours in the "dividable" cells (Stages I and II, II′). Some of the non-dividing cells (stage III, III′ and IV) may show a vestige of DNA-synthesizing ability, but if so, this is significantly decreased.

Consequently, the proportion of cells at any time capable of incorporating DNA label (percentage positive with e.g. [^{3}H]-thymidine) will be higher in stage I cells than in stage II cells (and perhaps higher in "pronormoblasts" than in "basophilic normoblasts"), and will be significantly lowered in stage III onwards.

The expected figures for positive percentage values would be:

"pronormoblasts"	about 70
"basophilic normoblasts"	about 50
stage II and II′ cells	about 33
stage III, III′ and IV cells	about 0–? 10

Furthermore, the grain counts (i.e. rate of uptake per cell per unit time) will be significantly lower in the occasional positive stage III, III′ and IV cells. This, in fact, agrees with the radioautographic findings in human bone marrows (Lajtha, 1959).

RNA, protein and haemoglobin synthesis all occur throughout the interphase, therefore at any given time all cells (apart from orthochromatic normoblasts) will show uptake of the respective precursors (100 per cent positive).

As, however, loss of RNA (cytoplasmic basophilia) starts by the end of stage Ic and is progressive through the stages afterwards, the uptake rate (per cell) of the respective precursors will be progressively less from stage II and II′ onwards.

^{59}Fe uptake

The first pronormoblast coming from the stem cell (before stage Ia) requires special consideration. It is not known what processes lead to the morphological transformation of a stem cell to pronormoblast, consequently, although immediately before division the rate of iron uptake may proceed at the "normal" rate (as in all stage I cells), some time before that, iron uptake may occur so slowly that it may not be visible on radioautographs. This would suggest that a small proportion of pronormoblasts (i.e. in the early part of the pre-Ia stage) will show no or very little ^{59}Fe uptake—and in fact 3 to 5 per cent of them are usually "negative" on radioautographs. Subsequently haemoglobin synthesis proceeds in every intact cell (except perhaps in cells in the process of dying in "ineffective erythropoiesis").

The rate of iron uptake per unit mass is taken as constant in any given stage, but the rate of uptake per whole cell will increase exponentially, in proportion with the increase in cell mass from $2n$ to $4n$. Although within one duplication the median or mean iron content of the cells is very similar, irrespective of whether the rate of synthesis is constant or exponential (e.g. content increasing from 10 to 20, mean value at linear rate = 15, at exponential rate = 14) (Fig. 2), nevertheless, the rate of iron uptake per cell is significantly less at the beginning of a stage compared with that at the end of the stage. For the same reason, "pronormoblasts" should have higher grain counts with ^{59}Fe than "basophilic normoblasts". Similarly, II′ cells (EPo no longer dividing), like all other stage I and stage I′ cells, synthesize haemoglobin at a faster rate near to the end of their cycle, and [illegible]

those over average basophilic normoblasts—in accordance with findings in rabbits and dogs (Suit *et al.*, 1957; Alpen and Cranmore, 1959)

The rates of iron uptake (haemoglobin synthesis) per $2n$ cell—i.e. immediately after mitosis—are taken as 0·5 μμg./hr. in stage I (a, b, c), 0·33 in stage II and II′, decreasing further in stage III from 0·25 to 0·2, and in stage IV from 0·17 to 0·10 μμg./hr. Stage III′ cells are $4n$ as they failed to divide, their rate is taken as falling from 0·5 (the rate at the end of stage II or II′) to 0·15 and further in stage IV′ from 0·12 to 0·10 μμg./hr.

The above rates of haemoglobin synthesis would result in blood reticulocytes containing 30 μμg. haemoglobin, a figure known to apply in man.

The haemoglob[illegible] content of the cells [illegible]
be [illegible]
15 [illegible]
[1954]), with a spread of hardly above 0 to 25. A concentration of

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significant reutilization of iron due to random destruction of reticulocytes or from "ineffective erythropoiesis") can also be calculated from Fig. 4. The curve thus obtained is illustrated in Fig. 5, the time at which 50 per cent of the eventually appearing radioactivity is released being about 74 hours; the curve is in good agreement with that obtained in man. It may be worth noting

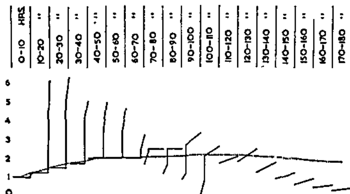


FIG 6. Grain counts over erythrocytes in peripheral blood after administration of ^{59}Fe as calculated from the model on Fig. 4

Dotted line: average grain count for *all* labelled cells at that time
Solid lines, proportional distribution of grain counts for cells released in a 10-hour period (i e between 20–30 hours 80% 1·5, 20% 5·1–6·3)
Note appearance of cells with high grain counts in the second and and third 10-hour period

that the rate of appearance between 20 and 120 hours is practically linear.

Similarly the grain counts over newly released cells can be calculated. The calculated grain count distribution and average grain counts over labelled peripheral red cells following labelling with ^{59}Fe are illustrated in Fig 6. It can be seen that by 30 hours some cells with high grain counts (from pathway II′) would be expected in the peripheral blood.

> 27 μμg. per 4*n* cell (or > 14 μμg. per 2*n* cell) is taken as the stage where "nuclear pyknosis" is initiated, and a content of about 30 μμg. as that at which the cells (reticulocytes or orthochromatic normoblasts) leave the marrow. The 2*n* nucleus is lost at the end of stage III (24·5 μμg. Hb per 2*n* cytoplasm), the 4*n* nucleus of the

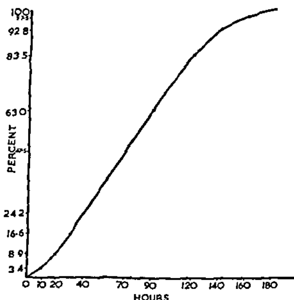


FIG. 5 Appearance of ^{59}FE in peripheral erythrocytes of man after a single intravenous administration of the tracer—as calculated from the model on Fig. 4. "Percent" = the proportion of the eventually appearing amount of radioiron

orthochromatic normoblast (IV′) at or after the time of release from the marrow.

The above concept also implies that a late IV′ orthochromatic normoblast takes up iron at a slower rate than an early IV marrow reticulocyte.

The rate of appearance of radioactivity in the peripheral blood after a single injection of high specific activity ^{59}Fe (assuming no

This postulated mechanism [illegible]
cel[illegible]
an [illegible]
pathway would produce only half as many cells as the II–III–IV pathway, but simultaneously with the shifting of more cells to II′ the increased erythropoietin level will increase the proportion of stem cells entering the erythron, thus increasing the absolute number of all erythroid cells.

Megaloblastic erythropoiesis

Although the interconversion of normoblastic and megaloblastic erythropoiesis is established, the biochemical "lesion" in the megaloblast is still unknown. There appears to be no deficiency whatever in these cells in respect of DNA, RNA, protein or haemoglobin synthesis. Nevertheless, in patients with megaloblastic marrows there is a significantly increased "ineffective erythropoiesis", i.e. increased cell death (Finch *et al.*, 1956).

A delay in entering mitosis has been suggested by Reisner as the explanation of the megaloblastic lesion (Reisner, 1958). While this hypothesis needs verification, such a delay may involve an elongation of the G_2 period (Fig. 2) thus increasing the proportion of cells with tetraploid amount of DNA, RNA and protein. This in fact seems to be the case in megaloblastic marrows. This delay, especially if coupled with a high proportion of "ineffective erythropoiesis", would result in a shift to the left, an increased erythropoietin level with concomitant shift to the II′–IV′ pathway i.e. orthochromatic nucleated red cells and possibly macrocytosis.

It is emphasized, however, that the nature of the megaloblastic "lesion" at the cellular level still awaits clarification, and the above theory is highly conjectural.

Radiation effects on the bone marrow

According to the model the bone marrow cells "proper" (in this case the normoblasts) are not a self-maintaining but a transient

Applications of the model

Regulation of erythropoiesis

In steady state equlibrium the proportion of stem cells differentiating into "pronormoblasts" is presumably regulated by erythropoietin. The mechanism of differentiation may be initiated by an enzyme induction in a proportion of the stem cells (perhaps similarly to adaptive enzyme formation).

Should the level of the circulating erythropoietin increase, the rate of "feed" of stem cells into the erythron will increase, thus increasing bone marrow erythroid cellularity, and, consequently, reticulocyte production. In experimental animals a tenfold increase of red cell production rate can be produced—this would imply a tenfold increase in the rate of feed of stem cells into the erythron.

With return of the erythropoietin level to normal the feed returns to the normal rate, and, as the rate of loss of nucleated erythroid cells is a first order process, the marrow cellularity will decrease until the loss and feed again reach equlibrium (Alpen and Cranmore, 1959).

Although the main action of erythropoietin is expected to be the enzyme induction in the stem cells, it is possible that it has a limited stimulating action on cells already containing enzyme—indeed *in vitro* studies indicated a slight (~ 10 per cent) stimulation of ^{59}Fe uptake. This stimulation, as mentioned before, will increase the proportion of cells in the II′ pathway, thus causing an apparent shortening of the stay of cells in the marrow at the expense of the number of cells produced. The result with ^{59}Fe would show (*a*) a steeper appearance curve in the peripheral blood (which is the case), (*b*) a higher than normal proportion of erythrocytes with high grain counts, and (*c*) a higher proportion of "orthochromatic normoblasts", both in the marrow and, at high erythropoietin levels, in the peripheral blood (as is frequently found after severe haemorrhage).

ate of the stem cells is artificially increased.* This would be chieved by increased erythropoietin levels (produced by vene-

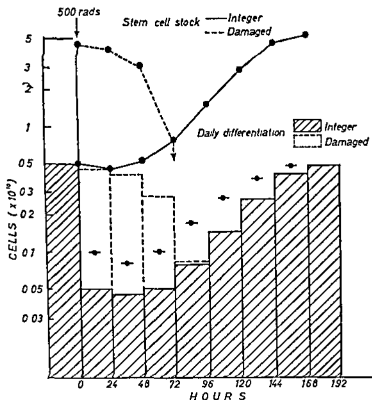


FIG 7 Numerical changes in stem cell population and stem cell differentiation after irradiation (from data in Table I).

section, anoxia, anaemic plasma injections), which, in fact, have been found to facilitate regeneration of erythroid elements after

* This is because, after certain doses of irradiation, e g 300–500 rads, it is mostly damaged cells which would be removed, either by differentiation into the erythron or by death in attempting division (Table I)

population; consequently, regeneration after radiation damage, for example, is effected by the stem cells (Lajtha, 1960). A merely temporary inhibition of mitosis would depopulate the bone marrow by inhibiting the efficiency of cell multiplication in the transient population (Fig. 4, Ia–III), without, however, inhibiting feed of stem cells into the erythron (Fig. 3). It is difficult to predict details of changes in cell population produced by radiation from such a model, as with small doses of radiation the inhibition of mitosis with uninhibited maturation leads to uncertainties in nomenclature. However, any interference with mitosis would lead first to a fall in the number of basophilic normoblasts, as in fact has been observed.

As the stem feed in man is postulated to be of the order of 5×10^9 cells per day, a store of stem cells in excess of this number is expected. A store of ten days' supply, i.e. 5×10^{10} cells, would mean only about 50 g. tissue, and a daily division of about one-tenth of the population.

A dose of radiation which would, for example, damage 90 per cent of the stem cells (of the order of 500 rads), would result in the following (illustrated in Table I and Fig. 7):

(*a*) Continued removal of damaged and undamaged stem cells (initially in the ratio of 9:1, as it is a random process) into the erythron, where most of the damaged cells eventually die.

(*b*) Regeneration from the undamaged fraction of the stem cell population, hampered by continued removal of undamaged cells into the erythron, i.e. slower than a theoretical exponential growth

If it is assumed that the stimulus for stem cell growth is "space" created by removal of cells from the population, then regeneration of the marrow cells would be expected to occur faster if, at an early time after radiation damage, for example, the removal

BONE MARROW CELL POPULATION FOLLOWING IRRADIATION

Depopulation of marrow due to mitotic inhibition and "G_1 delay" (Lajtha *et al*, 1958) with continued maturation ("first drop")

"Abortive recovery" of marrow with disappearance of mitotic delay and by "feed" of large number of damaged stem cells, which may be capable of 1–2 mitoses and/or differentiation

No change in blood reticulocytes up to 66 hours (assuming no death or hold-up in stage III and IV)

Blood reticulocytes start steep fall in numbers between 180 and 270 hours but recover to normal by 350 hours

higher than the absolute 20–30 per cent

TABLE I SCHEME OF EVENTS IN THE STEM CELL AND

Initial stem population: 5×10^{10} (normal maturation: $0{\cdot}5 \times 10^{10}$/day)

Stock 0 hour after 500 rads:	$4{\cdot}5 \times 10^{10}$ damaged	$0{\cdot}5 \times 10^{10}$ integer
Differentiation	0·45 „ „	0·05 „ „
No mitosis		
Stock at 24 hours	4·05 „ „	0·45 „ „
Differentiation	0·405 „ „	0·045 „ „
Mitosis	0·855 „ „	0·095 „ „
Stock at 48 hours	2·790 „ „	0·5 „ „
Differentiation	0·279 „ „	0·05 „ „
Mitosis	1·728 „ „	0·311 „ „
Stock at 72 hours	0·783 „ „	0·761 „ „
Differentiation	0·0783 „ „	0·0761 „ „
Mitosis	0·705 „ „	0·685 „ „
Stock at 96 hours	—	1·37 „ „
Differentiation	—	0·137 „ „
Mitosis	—	1·233 „ „
Stock at 120 hours	—	2·466 „ „
Differentiation	—	0·246 „ „
Mitosis	—	2·20 „ „
Stock at 144 hours	—	4·40 „ „
Differentiation	—	0·44 „ „
Mitosis	—	1·04 „ „
Stock at 168 hours	—	5·00 „ „

In every 24 hours 10 per cent of the stock is taken to differentiate, and sufficient divisions initiated to attempt to bring the total stock up to 5×10^{10}. It is assumed that the cells cannot divide more than once every 24 hours, and that the damaged cells die when attempting division and are removed from the population within 24 hours.

Note: If the proportion of cells differentiating is increased to 20 per cent (i.e. increased erythropoietin level) the removal of the damaged population is complete by 72 hours. Although this may delay the complete recovery of the stem population, it enhances recovery of the marrow "proper"—i.e. the number of integer stem cells differentiating into the erythron would increase

vided by "space" or "numbers", i.e removal of stem cells into the erythron

(*b*) Erythropoietin, whose production is primarily regulated by peripheral stimuli, randomly removes stem cells from the compartment.

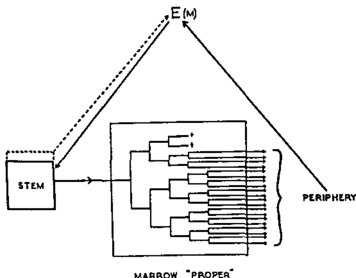


FIG 9 Scheme of kinetics of erythropoiesis
Peripheral conditions govern erythropoietin production (E); the erythropoietin concentration in turn determines the proportion of stem cells differentiating into the erythron (marrow proper) where they further multiply while differentiating

The interrupted lines indicate the hypothetical stimulation of erythropoietin production by increased stem compartment size

(M) signifies the hypothetical "myelopoietin"

(*c*) However, erythropoietin production may be stimulated independently by *another mechanism · increased stem "compartment size"* (Fig 9)

(*d*) Polycythaemia is primarily a slow proliferation of the stem cell population above the normal "compartment size". This first

irradiation (Stohlman, Cronkite and Brecher, 1955; Stohlman and Brecher, 1956).

Should the stem cells be "pluripotential" (i.e. precursors of erythroid, myeloid and megakaryocytic elements), then such simple postirradiation procedures may hasten regeneration of all bone marrow elements.

Polycythaemia vera

The most striking feature of polycythaemia vera (and also of chronic myelocytic leukaemia) is the fact that while the rate of regeneration from subnormal haemoglobin levels is fast (as it is in

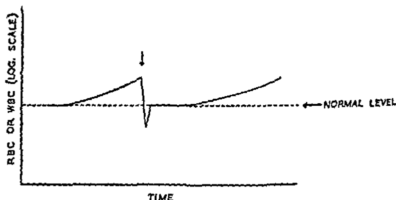


FIG. 8. Scheme of events in polycythaemia or chronic myelocytic leukaemia on treatment
Arrow indicates time of treatment (over-treatment, i.e. cell count sinking below normal level)

a normal subject), the rate of progress of the disease is slow (Fig. 8), allowing long periods of remissions following treatment. This is taken as an indication of a dual control of erythropoiesis (or myelopoiesis) in the disease, and prompts the following highly conjectural hypothesis:

(*a*) The stem cell "compartment" has its own homeostatic regulation mechanism, the stimulus for multiplication being pro-

colloid) may produce remission in both diseases; increased erythropoietin level in polycythaemia; and "myelokentric acid" in the urine of patients with chronic myelocytic leukaemia.

It is realized that invoking erythropoietin regulation by stem "compartment size" is a hypothesis which at present lacks experimental proof. Neither is good experimental evidence available which would help in identifying the stem cells. However, experiments in our laboratory, attempting to support some of the points raised here, are in progress and at a conference of this kind it may be permissible to theorize to some extent. Further, these latter hypotheses are in no way crucial for the model itself, which fits all the available experimental evidence, and which, it is felt, may help in understanding the dynamics of some haematological problems and suggest further experiments.

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results in an increased *number* of stem cells differentiating into the erythron (as a certain erythropoietin level removes a certain proportion of stem cells), but later represents a stimulus for increased erythropoietin production, which, by removing more stem cells from the compartment, tends to decrease its size, but further increases red cell production. The peripheral control of erythropoietin production (e.g. after haemorrhage) is unaffected.

(*e*) Any process which decreases stem cell population (e.g. radiation, massive venesections) would produce a remission, as the "over-production" of stem cells is a slow process in this disease.

(*f*) Stimulation of erythropoietin production by increased stem "compartment size" is not a particularly efficient process. If the stem cells were "reticulum" cells, this would explain the splenomegaly and hepatomegaly found in the disease.

(*g*) If the stem cells are pluripotential, then a similar stimulation of "myelopoietin" may be supposed. If stimulation of this factor is less sensitive than that of erythropoietin, then a mild increase of the stem compartment would only induce polycythaemia, while a more rapid increase may induce chronic myelocytic leukaemia—after an initial temporary polycythaemia state. This state, in fact, is frequently noticed in incipient cases of chronic myelocytic leukaemia.

(*h*) Finally, if polycythaemia and chronic myelocytic leukaemia are considered as results of a "benign" hyperplasia of the stem cells, then a "malignant" transformation of the stem cells (complete loss of differentiating capacity) would correspond to the acute blast cell leukaemias, and a partial loss of differentiating capacity may lead to the "subacute" promyelocytic or erythroleukaemias.

It is noteworthy that there is good evidence for "reticulum" hyperplasia (and not infrequently extramedullary haemopoiesis) in both polycythaemia and chronic myelocytic leukaemia; irradiation of the reticuloendothelial system alone (e.g. with ^{198}Au

Leblond: You don't know what a stem cell looks like and still you calculate that number? How?

Lajtha: We cannot juggle with this model very much because there are too many things which have to fit. If they do not fit then our model obviously is not right. As things are we think that one stem cell, whatever it is, will produce 20 reticulocytes. We know how many reticulocytes are produced per day, therefore we can calculate how many stem cells must be entering the erythron per day. We assume that there is more than one day's supply in the marrow and we quite arbitrarily chose ten days' supply. This would give around 4 per cent of stem cells in a marrow population.

If you look at the total erythroid cellularity and do a mitotic index or a stathmokinetic index you find a very significant increase when there is stimulation by erythropoietin or bleeding or hypoxia. This, however, does not necessarily mean that these cells divide more frequently! If you have a population which is not homogeneous (and very few cell populations are homogeneous in nature), and in which there are divisible and non-divisible cells, then a simple shift to the left, towards the divisible population, will increase the number of cells capable of division, and therefore the mitotic index and stathmokinetic index. So that for quantitatively measuring inter-mitotic cycle time the mitotic index and stathmokinetic index are only valid if you know you are dealing with a functionally homogeneous population of cells.

Leblond: But could you estimate the mitotic index of these various cell types? This would require identifying dividing cells. Could you identify them from the staining of the cytoplasm?

Lajtha: Not very reliably. But in any case even then they are not homogeneous, as you have disturbing factors, such as "ineffective erythropoiesis". There are cells (early forms!) which do not go into division. You do know that this particular population is practically disappearing under stress, because while normally not more than 75 to 85 per cent of the iron taken up by the marrow will come out, when [illegible]

stimulation of erythropoiesis will not die but will go on dividing and maturing.

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DISCUSSION

Yoffey: Dr. Lajtha, what is the estimated daily need for stem cells for erythropoiesis in human marrow?

Lajtha: The absolute number needed in a man is 5×10^9 for the erythroid series. We don't know how much for the myeloid series but at a guess I would say about the same.

Yoffey: Can you calculate what that is per mm.^{3} of marrow, because some of us have worked on animals?

Lajtha: It would imply something of the order of 3 to 4 per cent of the total marrow population being stem cells.

Leblond: Could you define the various cells you mentioned?

Lajtha: The pronormoblast is a largish cell (20 μ diameter) with a round nucleus, not necessarily central, a deeply basophilic cytoplasm, and a very finely stippled nuclear structure. The basophilic normoblast is very similar in every respect except that it is smaller, two-thirds to one-third in size, the nuclear chromatin is more heavily stippled and the nuclear/cytoplasmic ratio is altered, there being relatively less cytoplasm than in the pronormoblast. There is no sign of polychromasia with the Giemsa-Wright haematological stains. The early polychromasic normoblast may be the same size, or smaller, with a central or eccentric nucleus; the structure is more heavily clumped, very [illegible]

[illegible]

afraid it is essentially a question of religion where you draw the line between the stages after which you see no DNA labelling in the cells and no division.

Jacobson: Where are you putting the stem cells? Not in this series?

Lajtha: No. This series is completely unaffected by erythropoietin—it is completely unaffected in the animal when the animal is turning out five times as many erythrocytes as normal.

Table II (Cronkite)

MITOTIC INDICES (MITOTIC FIGURES/1,000 NUCLEATED CELLS) DETERMINED FROM COUNTS OF NOT LESS THAN 5,000 CELLS IN 21 DIFFERENT BONE MARROW ASPIRATIONS FROM 9 HEALTHY MALES

Individual	*Number of aspirations*	*Sum of mitotic indices*	*Mean of mitotic indices*
TF	7	61·4	8·77
EPC	5	40·5	8·1
VPB	2	18·9	9·45
LF	2	17·0	8·5
JB	1	8·4	(8·4)
VP	1	9·2	(9·2)
PH	1	9 0	(9·0)
GRS	1	10·3	(10·3)
SAK	1	8·0	(8·0)
	21	Mean 8 70 ± 1 88	Mean 8·86 ± 0·73

Table III (Cronkite)

DISTRIBUTION OF 650 MITOTIC CELLS FROM NORMAL BONE MARROWS ACCORDING TO CELL TYPE AND STAGE OF MATURATION

	Number of mitoses	*Relative frequency of mitoses*
Procrythroblast	31	1
Basophilic erythroblast	127	4·1
Polychromatic erythroblast	257 } 294	9·5
*"Orthochromatic" erythroblast	37 }	
Erythroblasts, total	452	
Myeloblast	18	1
Promyelocyte	36	2
Neutrophilic myelocyte	128	7 1
Neutrophil precursors, total	184	
Eosinophilic myelocytes	13	
Lymphoid cell	1	
Reticular cells	2	

* These mitotic cells have the size of polychromatic erythroblasts

Leblond: Would you say at what stage you think mitoses stop under strong "stimulation"? Could pronormoblasts go right through without any mitosis?

Lajtha: I don't think so. I think late basophilic normoblasts probably could go through—they could probably skip two divisions.

Leblond: Couldn't you trust the mitotic index for the first two, then?

Lajtha: No. You see this is not a question of skipping, it is cell death—cells which die in the marrow and which do not normally come out They do not take part in the normal dynamics of the marrow. But these cells somehow become more efficient under stimulation. Therefore you will get a different composition of the cell population

Cronkite: Dr. S. A. Killmann in my laboratory has sytematically studied for the last year the mitotic index in human bone marrow for each cell type. The results are shown in Tables I to VI (from a paper by Killmann, S. A., Cronkite, E. P., Fliedner, T. M., and Bond, V. P., submitted to *Blood*). I wonder whether these mitotic data are consistent with Dr. Lajtha's comments.

Table I (Cronkite)

COUNTS OF 1,000 NUCLEATED CELLS IN SEPARATE MARROW PARTICLES FROM THE SAME ASPIRATION FROM 5 HEALTHY MALES. ASPIRATIONS DONE BETWEEN 1 P M. AND 8 A M.

Individual	*Time**	*Slides counted*	*Mitotic figures/1,000 cells*
TF	1:20 p.m	5	7 0
TF	3:00 p.m.	6	6·5
TF	7.00 p.m.	5	10·8
TF	10·45 p.m	5	13·4
TF†	5.45 a m.	5	7·4
EPC	5:00 p m	5	8·2
EPC	10:00 p m.	5	6·0
EPC	10 45 p m.	5	5 8
EPC	11:00 p m.	5	11·8
VPB	10:20 p.m	6	10·5
LF	12·45 p m	5	7 8
JB	8:00 a m.	5	8 4
			$\bar{x}$ 8·63, S D. ±2·44

* In TF all marrow aspirations were done during one 24-hour period, in EPC, on different days

† At 10 a.m. the mitotic index was 8 8.

Table V (Cronkite)

Estimates of turnover times in human bone marrow (in hours)

		Author: Osgood[1]	Patt[2]	Fliedner[3] *et al.*	Mauer[3] *et al.*	Bond[4] *et al.*	Cronkite[4] *et al.*	Present[4] Report
Proliferating class.	Myeloblast	—	—	—	—	—	—	19·8–39·7
	Promyelocyte	—	12·5[5] (Promyelocyte–Myelocyte)	*ca.* 50[5]	72[6]	—	—	33·5–44·6
	Myelocyte	— —				—	—	58·3–64·1
Non-mitotable compartment.	Metamyelocyte	24 (Myelocyte–Metamyelocyte)	—	—	—	—	22	129·1–142
	Band	36	62·5	—	264	—	30·8	—
	Marrow segm.	—	—	—	—	—	35	—
Proliferating class;	Proerythroblast	—	5 75[5] (Proerythroblast–Basophilic erythroblast)	*ca* 22[5]	—	—	—	19·8–39·6
	Basophilic erythroblast	—			—	—	—	13·6–16·3
	Polychromatic erythroblast	48 (Polychromatic erythroblast–Orthochromatic normoblast)	—	—	—	—	—	10·8–11·5
Non-mitotable compartment;	Orthochromatic normoblast		—	—	—	≤19	—	15·5–16·6

[1] Based on bone marrow culture data and clinical observations of non-steady state situations.
[2] Based on mitotic indices
[3] Based on DF^{32}P labelling data
[4] Based on [^{3}H]thymidine labelling data
[5] Given as average turnover time of proliferating class.
[6] Given as average generation time of proliferating class
[5–6] As pointed out by Fliedner *et al* these figures must be multiplied by the number of successive divisions in the cell series to give the total average time spent by a cell going through the whole proliferating class.

Incidentally these data are consistent with the work Dr. Bond presented (1959. *In* The Kinetics of Cellular Proliferation, p. 188) on the turnover in the erythroid series, with a time of 22 hours for the turnover of the non-dividing compartment.

Lajtha: From the scheme you can easily read off the mitotic index, the lifespan in each compartment, or the total erythroid cellularity. If we assume roughly about one hour per mitosis, then we get the mitotic index either per compartment or per total erythroid cells—whichever you want to calculate.

The mitotic index, according to the model, is 14 erythroid mitoses per 1,000 nucleated red cells (21 mitoses in 1,517 "hours' worth"). This figure does in fact correspond well to the information you pre-

Table IV (Cronkite)

MEAN BONE MARROW DIFFERENTIAL COUNT OF 500 CELLS IN EACH OF 6 OF THE INDIVIDUALS STUDIED, RELATIVE COMPARTMENT SIZES, AND SPECIFIC MITOTIC INDICES

Cell type	*Number per 1,000 nucleated cells in bone marrow* Particle *smear*	*Relative compartment size*	*Relative Frequency of mitoses*[2]	*Number of cells in mitosis per 1,000 nucleated cells*[3]	*Specific mitotic index (%)*[4]
Myeloblast	10·0	1·0	1·0	0·252	2·52
Promyelocyte	33·7	3·37	2·0	0·503	1·49
Myelocyte	163·0	16·3	7·1	1·788	1·097
Non-mitotable marrow[1] granulocytes	361·4	36·14	0	0	0
Proerythroblast	16·7	1·0	1·0	0·422	2·53
Basophilic erythroblast	35·0	2·1	4·1	1·729	4·94
Polychromatic erythroblast	70·7	4·23	9·5	3·998	5·65
Orthochromatic normoblast	102·0	6·11	0	0	0

[1] Metamyelocytes to marrow segmented neutrophils.
[2] From Table III.
[3] Calculated from relative frequency of mitoses on different maturation levels and 2·54 total neutrophil and 6·15 total erythroblast mitoses per, 1,000 nucleated cells.
[4] Specific mitotic index = percentage of cells in compartment in mitosis at any time.

animal with the more rapid maturation time? You say that in the human marrow with a total maturation time of 180 hours you needed 3 to 4 per cent of stem cells in the marrow. What is your comparable figure for a small animal, a rabbit or rat, which has a more rapid maturation time?

Lajtha: It is not only the maturation time which matters but also the relative lifespan of the mature cell.

Yoffey: What I am getting at is this: the relative lifespan of the red cells in many of our small laboratory animals is about half that of the human red cell or even less. Other things being equal, that at least doubles the stem cell requirements. But in addition, the actual maturation period in the marrow is appreciably shorter.

Lajtha: It does not necessarily double the stem cell compartment size. It may double the turnover time of the stem cell compartment cells. We assume for man a ten-day supply and therefore one-tenth of them divide per day. In an experimental animal with the same ten-day supply but thirty per cent dividing per day, you could get three times the production for the same compartment size. So size is not the only determining factor. Turnover time of the compartment is in a sense more important.

Yoffey: That is in fact an assumption. You can say either you have the same number of stem cells with a larger proportion turning over, or if you have the same proportion turning over you would need a larger number of stem cells?

Lajtha: Yes.

Jacobson: Do the cells as you see them when you are using erythropoietin as a stimulus contain a normal amount of haemoglobin?

Lajtha: Yes.

Jacobson: What is the maximum stimulation possible?

Lajtha: Probably eight times, which is what we have seen so far.

Jacobson: But there is a maximum?

Lajtha: Yes, I think so.

Jacobson: You told me you probably had the explanation for the abortive rise in the irradiated animal. I think this is a very important thing.

Lajtha: You produce a temporary inhibition of mitosis by irradiation. This will immediately stop mitosis in the marrow compartment proper

Table VI (Cronkite)

QUANTITATIVE ESTIMATES OF ERYTHROPOIETIC AND GRANULOCYTOPOIETIC CELL MASS IN STANDARD MAN

Author	*Erythroblasts* (cells per kg. × 10^9)	*Neutrophils and precursors* (cells per kg. × 10^9)
Osgood	8·6	25·7*
Patt	3·4	8·3
Suit	4·6	—
Donohue *et al*	5·0	11·4
Mauer *et al.*	—	12·5
Present Report	4·5	10·7

* Segmented forms excluded.

sent, Dr. Cronkite (in Tables II and IV). You give an overall mitotic index of about 8·8 per 1,000 marrow cells, which is composed of 2·54 neutrophil mitoses and 6·15 erythroid mitoses. Well, 6·15 erythroid mitoses per 1,000 *bone marrow cells* would give something of the order of 16 to 18 mitoses per 1,000 *nucleated red cells* (as the latter constitute about one-third of the total marrow cellularity).

Considering the nature of our calculations and the error involved in mitotic indices, I think our calculated and your measured values are in remarkably good agreement

Braunsteiner: Dr. H. Weicker has a scheme based on calculations where he had to postulate the further division of the reticulocyte. What is your opinion about this point?

Lajtha: We cannot find any evidence for it—we tried with ^{59}Fe grain count halving, several times, and that certainly would have shown it, had it existed.

Yoffey. We read Weicker's work (1954. *Arztl. Wschr.*, 9, 1017) with great care though it was rather ambiguous. On one occasion he had a homoplastic division of the stem cell. On another occasion he [illegible] wavering on

[illegible]

[illegible] so that we will get the quantitative background right, would you give us your opinion of the relative stem cell requirement in the smaller

We have recently attempted to induce polycythaemia with varying amounts of erythropoietin, as measured by the fasted rat assay. When plasma from animals exposed to altitude was used as the source of erythropoietin, we were unable to induce polycythaemia even though the plasma increased the ^{59}Fe incorporation in the fasted assay from a control level of 6 per cent to 21 per cent. When more erythropoietin was given, roughly threefold, a substantial polycythaemia resulted in the intact animal, the red cell mass rising from 4·9 ml. to 6·9 ml. Thus you must give a great deal of erythropoietin before actually seeing an increase in red cell mass Apparently, there is a mechanism for "dampening" the effect of smaller amounts Accordingly I wonder if the fine control of red cell production can be explained on the basis of erythropoietin. Is it not more likely that another mechanism, perhaps feedback from the peripheral red cells, is responsible? In this regard there are patients with hereditary spherocytosis who very frequently have a normal haemoglobin and haematocrit. We presented data on one such patient in whom we did a 40 per cent exchange transfusion. Whereas the patient had been making red cells at a rate five times normal before the transfusion, immediately following the transfusion there was a substantial decrease in the rate of erythropoiesis, though the haemoglobin remained constant. At the moment we have a youngster who had a splenectomy and has 1,000,000 reticulocytes/mm.^{3}, 10 g. of haemoglobin, and a three-day apparent chromium half-time. I have transfused him up to 14 g. of haemoglobin and he continues to make an increased number of red cells, though fewer than before, the reticulocyte count being 400,000/mm.^{3}, which is roughly eight times normal. So it seems there is a feedback mechanism which is not explained by hypoxia and erythropoietin.

Lajtha: The scheme as presented does not necessarily imply that there is only one erythropoietic stimulation. The point which is important is that the mode of stimulation of erythropoiesis, unless proved otherwise, is not through the erythroid compartment itself, but through the stem compartment.

Stohlman: Why can't a decrease in the death rate or "ineffective erythropoiesis" of the erythroid compartment be used for fine regulation of cell production? This would take care of small amounts of blood loss, i e. 2 to 3 ml.

and start a depopulation because maturation is not inhibited, i.e. the cells are still pouring out. This inhibition of mitosis, however, is temporary and will recover before the compartment is completely emptied. As soon as mitosis recovers there will be an increase in numbers straight away. Nevertheless, this will result in one or two mitoses only, because the vast majority of the cells will eventually die, and you get a secondary and deeper depopulation which only later will be filled by regeneration from the integer fraction of the stem cells.

Stohlman: We have previously postulated maturation with and without division (Stohlman, F., Jr. [1959]. *In* The Kinetics of Cellular Proliferation, p. 318) and I think Dr. Lajtha's scheme differs only in quantitative details from ours (cf. Fig. 1). I think that under maximum

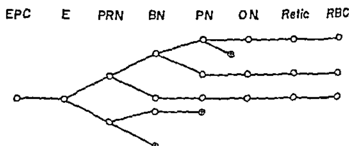


FIG 1 (Stohlman) Schematic representation of red cell proliferation. EPC = early progenitor cell, E = erythroblast; PRN = pronormoblast, BN = basophilic normoblast; PN = polychromatophilic normoblast, ON = orthochromatic normoblast; Retic = reticulocyte, RBC = red blood cell. (From Stohlman, F., Jr. [1959]. *In* The Kinetics of Cellular Proliferation, p 323. New York: Grune & Stratton)

stimulation in a person, following severe haemorrhage or severe hypoxia, most of the increase in red cell production would result from the production of erythropoietin and the influx of cells from the stem cell compartment as Dr Lajtha has described. However, it seems to me very difficult to accept the fact that erythropoietin is the sole regulator, and data have been presented suggesting the dual regulation of erythropoiesis (Stohlman, F., Jr. [1959]. *Ann. N.Y. Acad. Sci.*, 77, 710).

HUMORAL INFLUENCES ON BLOOD CELL FORMATION AND RELEASE*

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CONVINCING experimental evidence, amassed over the past ten years, has supported the existence of an erythropoietic-stimulating factor (ESF or erythropoietin) in the plasma or urine of animals subjected to anaemic and hypoxic anoxia as well as in the body fluids of anaemic human subjects (Erslev, 1953; Borsook *et al.*, 1954; Gordon *et al.*, 1954; Hodgson and Tohá, 1954; Plzak *et al.*, 1955; Linman and Bethell, 1956; Stohlman and Brecher, 1956; Mirand, Prentice and Slaunwhite, 1959) It is of interest that thus far only blood and urine have served as reliable sources of the ESF and attempts to extract the factor from a variety of organs have, in the main, proved unsuccessful (Gordon, 1959*a*). The significance of its presence in only the body fluids remains to be determined. One hypothesis analogizes the production of the ESF to that of the pressor factor, angiotensin (Gordon, 1959*a*).

Our laboratory has been concerned recently with a study of the biological actions and chemical properties of the urinary ESF obtained from children with thalassaemia major (Winkert *et al*, 1958*a*, *b*; Gordon *et al*, 1959*b*, *c*). Such urine, when secured from severely anaemic patients, has proved a consistent source of high activity In addition, as an extraction source, urine offers the

* This investigation was supported by research grants from the National Heart Institute of the National Institutes of Health, U S Public Health Service (H-3357) and the Damon Runyon Memorial Fund for Cancer Research (DRG-406).

Lajtha: It could. But there is a limit, as ineffective erythropoiesis is normally not more than 10 per cent.

Stohlman: I am not referring to major blood loss—certainly not the loss of 10 per cent—but for making up 2 or 3 ml. per day.

Astaldi: I am pleased that Ferrata's theory has received further confirmation this morning from your lecture, Dr. Lajtha. I should like to mention two biological situations which may confirm that without the stem cell compartment, erythropoiesis cannot continue, despite the proliferation of the erythroblast. The first situation is the following: when you explant a fragment of bone marrow *in vitro*, the erythroblastic series finishes in a few days, despite the proliferation of erythroblasts, because the stem cell series go first into macrophagic evolution and secondly into histiofibroblastic evolution. The second condition is: when you evaluate the proliferation activity of the erythroblast in the vessels of chick and other embryos during the first series of erythropoiesis, the megaloblastic one, you may obtain very high proliferative indices. For instance, on the second day of development in chick embryo, you have a stathmokinetic index which is about 500 mitoses in 1,000 cells. Despite this very high degree of proliferation all the erythroblasts reach maturation in a few days and erythropoiesis does not go on longer in the vessels, where there are no stem cells.

Stohlman: What happens if there is a change in the concentration of haemoglobin in your scheme, Dr. Lajtha, such as one sees in spherocytosis or spheroid insufficiency?

Lajtha: I don't really know. We postulated, to be simple and unsophisticated, a regulation by haemoglobin concentration in the cytoplasm. It may be more complicated than that—perhaps not the haemoglobin concentration but the concentration of X.

least squares (Mode, 1941). Equation (1) describes the regressi of the reticulocyte responses (y) in recipient rats upon the haem globin levels (x) of the donor patients.

$$(1) \qquad y = -2 \cdot 1x + 13 \cdot 2$$

Equation (2) represents the regression of the haematocrit sponses (z) in the recipients upon the donor haemoglol values (x).

$$(2) \qquad z = -1 \cdot 8x + 11 \cdot 1$$

When these two equations are solved simultaneously, equati (3) for y as a function of z is obtained.

$$(3) \qquad y = 1 \cdot 16z + 0 \cdot 3$$

Since the intercept 0·3 is of small magnitude, it may neglected. Because the factor 1·16, within the limits of scatter the data available, is approximately 1, the equation may simplified to $y = z$. With this convenient approximation, perce age haematocrit and percentage reticulocyte responses (obtain on the sixth day) may be regarded as equivalent, and can either averaged or added. The value obtained by the addition the rise in percentage haematocrit and the increase in percent of reticulocytes is referred to as the H-R unit. This method affo the convenience of a one-parameter unit, while retaining some the assurance of the two. The real virtue arises from the f that use of the H-R unit results in an elimination of reticuloc increases which stem from effects that lower the haematoc Reliance upon reticulocyte counts, or ^{59}Fe uptake methods alo can lead to the false impression that certain substances direc stimulate erythropoiesis when in fact they may be haemoly Haemolytic effects have actually been observed in eight out of 34 urine samples assayed. On the average, the reticulocyte a haematocrit changes, for these haemolytic specimens, are eq and opposite. Addition of the haematocrit decrement to

antage of being easily collected and relatively free of blood teins. It is the purpose of this ... ierimenta ... stence ... inducing factor (LIF).

Factors determining the appearance of the ESF in urine

uman urine

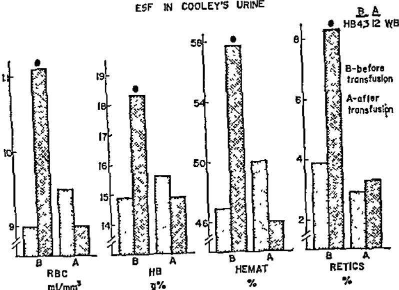
We have recently reported the existence of a positive correlan between the intensity of the anaemia in thalassaemia subjects d the presence of the ESF in their urines (Gordon *et al.*, 1959*b*). ibility to demonstrate this relation on previous occasions was e to the non-availability of sufficient numbers of samples from ensely anaemic subjects. Up to now, the erythropoietic effects 34 urine specimens * from a total of 13 subjects with thalasemia major have been examined.

The assay routinely employed for screening urine activity conts in injecting, subcutaneously, groups of three to five adult male rats of the Long–Evans strain daily for five days, with 3 ml. filtered but otherwise untreated urine. Changes in the perieral reticulocyte and haematocrit values are recorded on the th day. We have found it useful, in our screening procedures, employ as an erythropoietic index the sum of the change in rcentage of reticulocytes and ... ...olute magnitude.

...attempt was made to determine the mathematical equivalence of the reticulocyte and haematocrit responses to the 34 urine pecimens tested. Two equations were derived by the method of

* The urine specimens should be frozen as quickly as possible after collection. n most cases, arrangements have been made to collect the urines in containers urrounded by dry ice. In our experience, this has proved more successful n ensuring retention of activity in the urine than the use of preservatives or ntibiotics.

reticulocytosis but no haematocrit change. The H-R unit, when applied in such cases, tends to cancel out haemolytic effects and thus affords a more realistic evaluation of the actual amounts of ESF present.



second bar indicates that the value is significantly different ($P < 0.05$) from the initial value (first bar) Haemoglobin values of the patient before (B) and after (A) transfusion with 1,500 ml of compatible whole blood (WB) over a two-day period are indicated on the upper right

The unit responses obtained with the 34 human urine specimens are plotted in Fig. 1. The line constructed by the method of least squares indicates that the ESF usually appears in detectable amount in the urine when the haemoglobin values in the patients are approximately 5 g. per cent. Increasing severities of anaemia

reticulocyte rise tends to produce a cancellation and a near-zero H-R unitage. It is to be anticipated that a mixture of haemolytic

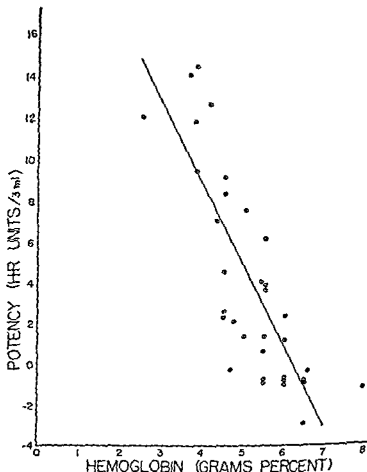


FIG 1 Potency of urine in H-R units, per daily dose of 3 ml. for 5 days, as a function of the haemoglobin levels in children with thalassaemia major

and direct stimulatory effects will occur in other cases In these, it would not be an economical procedure to discard samples that were contaminated with just enough haemolytic material to evoke

at the Umbrian Farms in Sparta, New Jersey, male sheep weighing approximately 40 kg were injected daily with a starting dose of phenylhydrazine equivalent to 5 mg. per kg. of body weight. After the tenth injection, the dose was varied somewhat according to the physical and haematological condition of the sheep. Urine was collected through a funnel strapped about the partially suspended animals and was led through a tube into a polyethylene bottle where it was frozen immediately. Before treatment, the sheep haematocrits averaged 33 per cent. After ten injections, the haematocrits had fallen to approximately 10 per cent. Urine collections were now begun and phenylhydrazine injections were temporarily discontinued. The haematocrit values rose to approximately 15 per cent in the next six days. Injections were now reinitiated and, after 16 days, the haematocrits fell below 9·5 per cent. The sheep died after the 20th injection of the second series with haematocrits in the range of 7·0–8·5 per cent.

The urines were assayed in the usual way in intact adult female rats (i.e. 3 ml. per day for five days). The responses, in terms of H-R units, are plotted in Fig. 3. It is to be noted that erythropoietic-stimulating activity does not appear in the urine when the haematocrit values of the sheep are above 10 per cent. A sharp increase in the urinary excretion of the factor is seen when the haematocrits have fallen below the 7·5 to 8·0 per cent levels. This may represent a marked increase in the rate of production of the ESF and/or a lowering in the renal threshold for its excretion. It is of interest that good yields of the ESF in the plasma of phenylhydrazinized sheep are obtained when their haematocrits range between 10 and 15 per cent (White *et al.*, 1960) and in the plasma of dogs bled to haematocrits below 12 per cent (Naets, 1959). It thus seems likely that significant quantities of the factor are not detectable in the urine until the haematocrits in the donor phenylhydrazinized sheep have fallen below 10 per cent. Haematocrits of 9 per cent or less must also be attained for the ESF to appear in the urine of repeatedly bled dogs (Naets, 1959).

are associated with a linear increase in the quantity of the factor in the urine.

The ESF disappears quickly from the urine following transfusions of either whole blood or packed red cells. A typical case is presented in Fig. 2. Urine samples were obtained from a five-year-old boy with Cooley's anaemia before and after transfusion. The Hb level in the child before transfusion was 4·3 g. per cent and, after a two-day transfusion period with 1,500 ml. of whole blood, it rose to 12·0 g. per cent. Daily 3 ml. subcutaneous injections of pre- and post-transfusion specimens of urine were made, for ten days, into two groups of five intact adult female rats. The presence of strong erythropoietic-stimulating activity in the pre-transfusion urine is indicated from the highly significant increases in red cell counts, haemoglobin, haematocrit and reticulocyte values in the recipient rats. Also noted is the absence of demonstrable activity in the urine on the day following the last transfusion A similar situation exists for the ESF content of pre- and post-transfusion samples of plasma from Cooley's anaemia children (Medici *et al.*, 1957). The speed with which the factor disappears from both the plasma and urine is probably the result of (1) a prompt suppression by the transfused cells of ESF manufacture, and (2) a rapid utilization of the available circulating material during the transfusion period.

Sheep urine

White and co-workers (1960) have recently found consistent erythropoietic-stimulating activity in the plasma of sheep made anaemic with phenylhydrazine and have indicated a direct relation between the intensity of the anaemia in the sheep and the level of their circulating ESF. It seemed of interest to determine, in this species, the degree of anaemia necessary for the appearance of the factor in the urine. To this end, a collaborative study on sheep urinary ESF was undertaken with Dr. Milton Eisler of the Schering Corporation. In the experiments to be reported, conducted

conditions, the quantity of ESF in the urine of thalassaemia subjects may often approach that detectable in similar volumes of whole plasma from the same patients.

Normality of the red blood cells produced in response to injections of the urinary ESF in rats

The appearance of abnormal red cells has been reported following injections of plasma filtrates from phenylhydrazinized rabbits or from polycythaemic patients (Linman and Bethell, 1956, 1957). It seemed critical to determine whether this was also characteristic of the RBC population after injections of the human urinary ESF. The possibility exists that the urinary material exerts a direct toxic action on the circulating RBC, rendering them non-functional, a reaction which would then evoke secondarily the production of endogenous ESF within the recipients.

Preliminary experiments have established a normal osmotic fragility for the RBC of rats treated with urine from Cooley's anaemia subjects (Gordon *et al.*, 1959*a*). More recently, we have extended these experiments to include a larger series of urine samples In general, similar results have been obtained with the photometric method for estimating the degree of haemolysis. Direct cell enumeration techniques in the haemacytometer have also failed to reveal a decrease in the osmotic resistance of the RBC of rats treated chronically with active urine. In addition, these RBC contained electrophoretically normal haemoglobin and normal concentrations of alkali-resistant rat haemoglobin (Gordon *et al.*, 1959*b*).

Additional evidence for the competence of the RBC is indicated from their oxygen content and capacity. In the experiments designed to test these parameters, six adult female rats received 18 subcutaneous injections of 3 ml of active urine from a child with Cooley's anaemia over a period of 21 days; six rats from similar

Calculations indicate that a greater degree of anaemia is generally required for the appearance of the factor in the urine of phenylhydrazine-treated sheep than in the Cooley's anaemia

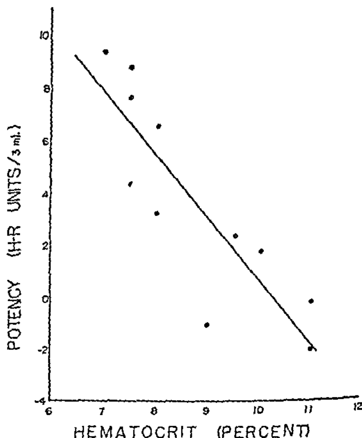


FIG 3. Potency of urine in H-R units, per daily dose of 3 ml. for 5 days, as a function of the haematocrit level of sheep rendered anaemic with phenylhydrazine The line was established by the method of least squares.

subject. In order to obtain large titres of the ESF in urine, attempts should be made to bring the haemoglobin or haematocrit values to approximately one-quarter to one-fifth of normal. Under these

conditions, the quantity of ESF in the urine of thalassaemia subjects may often approach that detectable in similar volumes of whole plasma from the same patients.

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litters served as untreated controls. The urine-injected rats were rendered polycythaemic by this treatment, exhibiting mean haematocrit values of 63·4 per cent and reticulocyte counts of 4·9 per cent as compared to haematocrits of 46·9 per cent and reticulocytes of 1·7 per cent in the controls. On day 22, both the treated and control rats were lightly etherized and 5 ml. of blood were withdrawn from the dorsal aorta into heparinized oiled syringes. Haemoglobin determinations were made photocolorimetrically by the acid haematin method. The blood was divided into two samples: in the first, the oxygen content was determined immediately; the second sample was introduced into a 10 ml. micro-suction flask and equilibrated with 100 per cent oxygen for approximately five minutes, following which the oxygen-saturated blood was drawn into an oiled syringe and its oxygen content determined. All determinations were conducted in micro-Scholander pipettes using the method of Roughton and Scholander (1943) and at a mean barometric pressure of 766 mm. Hg and a mean temperature of 24°. These values were then corrected to standard temperature and pressure.

Table I

ARTERIAL OXYGEN CONTENT AND CAPACITY OF RBC FROM RATS TREATED CHRONICALLY WITH HUMAN URINARY ESF (MEAN VALUES ± S.E. OF MEAN)

	(*a*) O_2 *Content* (*b*) O_2 *Capacity* (vols per cent)	*Hb* (g per cent)	(*a*) O_2 *Content* (*b*) O_2 *Capacity* (ml. O_2/g Hb)
Urine-treated	(*a*) 20·0 ± 0·98	16·7 ± 1·05	(*a*) 1·20 ± 0·063
	(*b*) 24·7 ± 1·12		(*b*) 1·49 ± 0·055
Controls	(*a*) 15·5 ± 0·39	12·6 ± 0·24	(*a*) 1·23 ± 0·055
	(*b*) 19·3 ± 0·81		(*b*) 1·54 ± 0·071

Table I indicates normal arterial oxygen-carrying capacities and saturations for the RBC of rats treated with active urine. Thus, there was no observable impairment in the ability of the RBC of ESF-treated rats to pick up oxygen, either during the short exposure to pulmonary oxygen *in vivo*, or during prolonged

exposure of the RBC to pure oxygen *in vitro*. These data provide further evidence for the contention that the RBC evoked by the human urinary ESF constitute a functionally competent population of cells. More recently, Van Dyke and Berlin (1960), employing *in vivo* [2-^{14}C]glycine techniques, have detected no differences in the lifespan of RBC from rats treated chronically with human erythropoietin and from untreated controls.

Further studies on the extraction and chemical properties of the human urinary ESF

Two methods have been employed in our laboratories for the extraction of the human urinary ESF. The first utilizes a modification of the kaolin-adsorption procedure described by Albert (1956) for extraction of human urinary chorionic gonadotrophin. Full details of this method have been provided elsewhere (Gordon *et al.*, 1959*c*) and only brief mention of some of its features need be given here. The human urinary ESF was adsorbed on kaolin at pH 4 8. Serial elution was carried out in the alkaline range with ammonium acetate buffers of increasing pH and with 1M-NH_4OH. Neutralization of the eluates was performed either with 1N-HCl or by dialysis in Visking casing against an 0·1M-sodium phosphate buffer at pH 6·8. Bioassays in intact rats indicated that the major activity could be accounted for in the pH 8 and 1M-NH_4OH eluates In a typical run (kaolin run 3) a 230-fold purification of the factor was achieved in the 1M-NH_4OH eluate. Administration of 300 µg. of this eluate daily for five days caused a significant enhancement of erythropoiesis in intact rats. Using a more sensitive assay, namely radioiron incorporation into the RBC of starved rats, this preparation would be found to be active at the 60–75 µg level Hodgson and co-workers (1960), Rambach (1959) and Slaunwhite (1959) have recently confirmed the efficiency of the kaolin-adsorption, alkali-elution procedure in the extraction of the urinary ESF.

litters served as untreated controls. The urine-injected rats were rendered polycythaemic by this treatment, exhibiting mean haematocrit values of 63·4 per cent and reticulocyte counts of 4·9 per cent as compared to haematocrits of 46·9 per cent and reticulocytes of 1·7 per cent in the controls. On day 22, both the treated and control rats were lightly etherized and 5 ml. of blood were withdrawn from the dorsal aorta into heparinized oiled syringes. Haemoglobin determinations were made photocolorimetrically by the acid haematin method. The blood was divided into two samples: in the first, the oxygen content was determined immediately; the second sample was introduced into a 10 ml micro-suction flask and equilibrated with 100 per cent oxygen for approximately five minutes, following which the

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	(*b*) 24·7±1·12		(*b*) 1·49±0·035
Controls	(*a*) 15·5±0·39	12 6±0·24	(*a*) 1·23±0 055
	(*b*) 19·3±0·81		(*b*) 1·54±0 075

oxygen-saturated blood was drawn into an oiled syringe and its oxygen content determined. All determinations were conducted in micro-Scholander pipettes using the method of Roughton and Scholander (1943) and at a mean barometric pressure of 766 mm. Hg and a mean temperature of 24°. These values were then corrected to standard temperature and pressure.

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be noted that preparation A was the most active of the three, with a daily dose of 180 μg. over five days significantly stimulating erythropoiesis in the intact rat. The dialysed urinary ethanol precipitate displayed a lower specific activity which, however, was greater than that seen with the CS-1 preparation of Borsook.

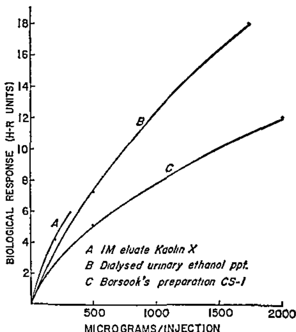


FIG 4 Erythropoietic responses of rats, in H-R units, to three different preparations of the ESF as a function of the daily dose in μg

Interpolation of the dose-response curves (Fig. 4) indicates that the CS-1 preparation contained 8 0 H-R units per mg. Since Goldwasser (1959) found that the CS-1 preparation possessed 10·4 cobalt units per mg., it may be estimated that one H-R unit is equivalent to 1·3 Goldwasser cobalt units. We have not as yet assayed the CS-1 standard by the starved rat procedure. However,

Another method we have employed has consisted of ethanol precipitation of the urine followed by prolonged dialysis of the precipitate. In a typical run, 1,600 ml. of absolute ethanol were added to 400 ml. of filtered active urine. The additions were made slowly at temperatures ranging from $-3°$ to $-10°$. Ten hours were allowed for the precipitate to form at $-10°$. The precipitate was spun down in the cold, the alcohol supernatant decanted and the precipitate dissolved in 150 ml. of 0·08M-NaCl. This material was now dialysed in 8/32″ Visking casing against 0·16M-NaCl-0·025M-PO_4 buffered at pH 7·5. The dialysis was performed over a period of four days at 5° to 10° in an improvised rocking trough. Each day for three days 450 ml. of dialysate were replaced by fresh solution. On the fourth day, the buffer solution used was 0·16M-NaCl but only 0·006M in PO_4. The dialysand was lyophilized to dryness and a light tan-coloured powder was obtained. Calculations indicated that a recovery of 49·2 per cent was achieved with ethanol precipitation alone, 44·0 per cent with ethanol precipitation followed by dialysis and 48·9 per cent with the ethanol precipitation-dialysis-lyophilization procedures. These differences are not significant. Additional studies showed that recovery of activity was only 28 per cent when ethanol precipitation was conducted at pH 4·8 and approximately 40 per cent when carried out at pH 6·1 or 8·5. Raising the concentration of ethanol from 33 per cent to 80 per cent resulted in increasing yields of the factor.

Fig 4 compares the erythropoietic responses, in terms of H-R units, to different quantities of three preparations containing the ESF. "A" represents a tenth run of a 1M-NH_4OH kaolin eluate of Cooley's anaemia urine dialysed against 0·16M-NaCl for 48 hours; "B" is a dialysed ethanol precipitate of Cooley's anaemia urine prepared by the method described above and "C" is a dialysed water-extracted ethanol precipitate of a boiled plasma filtrate from phenylhydrazinized rabbits (preparation CS-1) kindly supplied by Dr. Henry Borsook and his associates. It will

but there is a shift of usually 5 to 10 mμ to the right in the absorption spectra of the ethanol-precipitated urinary material. More significant, however, is the greater height at all points of the curve for the urinary material. The 280/305 mμ optical density for the CS-1 preparation is 4·7 and that for the urinary material shown in Fig. 5 is 1·4. The absorption between 300 and 400 mμ

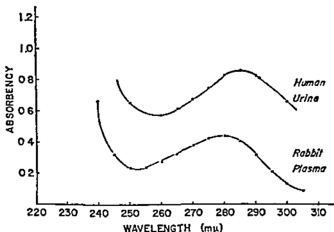


FIG 5 Ultraviolet absorption spectra of a human urinary and a rabbit plasma ESF preparation. A—Thalassaemia major urine-ethanol precipitation followed by dialysis for 4 days, 0·36 mg/ml. B—Plasma from phenylhydrazinized rabbits (Borsook's CS-1)—dialysed, water-extracted ethanol precipitate of boiled plasma filtrate, 0 67 mg/ml.

is probably an indication of contaminants including some of the pigment associated with the urinary ESF. Reprecipitation of the urinary preparation with ethanol or ammonium sulphate did not result in a significant elevation of this ratio. Although the kaolin eluates of human urine have generally proved to be more highly purified preparations (absorption peak at 280 mμ) than those obtained with ethanol precipitation (absorption maximum at 285 to 290 mμ), more consistent recovery has been achieved with the

three human urinary ESF preparations with established H-R unitage were tested by the radioiron method. We have found that, on the average, about 3·6 H-R units, or 4·7 cobalt units, per injection are required to produce a doubling in the ^{59}Fe uptake in our modified starved rat assay.*

It is apparent from Fig. 4 that analysis of any preparation containing the ESF should consider the activity at more than one dose level. The nature of the three curves makes it seem likely that the intensity of the erythropoietic effects noted with the higher doses of human urinary preparations cannot be reproduced with any quantity of plasma material. This may signify that (1) the urinary ESF is a more potent and somewhat chemically different molecule than that derived from plasma, (2) the plasma preparations thus far employed are associated with greater quantities of tenacious erythropoietic inhibitory substance(s) than is the case for urine, or (3) the plasma factor exerts additional side effects which prevent its maximal erythropoietic activity from being attained. The possibility requires testing that the ESF may take a somewhat different form depending on the body fluid and the clinical state in which it is found.

On the other hand, the probability that the ethanol-precipitated dialysed human urinary preparations and similarly prepared rabbit plasma material share some basic properties is seen from the nature of the ultraviolet absorption curves (Fig 5). It may be noted that the general shapes of the two curves are alike,

* Five μmole doses of Co^{++} have been found by White and co-workers (1960) [illegible] established as

Table II

RELATIVE PURITY OF ESF PREPARATIONS

	Specific activity		Carbohydrate (per cent of total protein + carbohydrate)	Overall yield (per cent)
Human urine	H-R units/mg.	Cobalt units*/mg.		
1. Raw urine (runs 4 and 6)	0 50	0·60	—	100
2 EtOH ppt (run 9)				
(*a*) not dialysed	2·50	3·30	—	47
(*b*) dialysed	10 00	5·50	29	48
3. 1M-NH_4OH kaolin eluate				
(*a*) run 3	12·00	16·00	23	12
(*b*) run 10	21·00	27·00	8·9	11
Sheep Plasma				
(White *et al*, 1960)				
1 Raw Plasma	0·005	0 007	—	100
2. Step I (DEAE)	0·780	1·000	—	63
3 Step II (IRC 50)	1·200	1·550	—	49
4 Step III (DEAE)	1·700	2·230	—	22
5. Step IV (IRC 50)	15–80	20–100	8·2	—

* As defined by Goldwasser (1959).

latter method. In several runs with the anion exchanger diethylaminoethyl (DEAE)-cellulose, it was noted that considerable amounts of the pigment in the ethanol-precipitated dialysed urinary material clung to the tops of the columns and was not eluted over a wide range of pH values. This suggested that DEAE might be used to eliminate the pigment from the urinary extracts. Batch operations, using DEAE at pH 7·4, at which the ethanol-precipitated dialysed urinary ESF is not appreciably adsorbed, resulted in removal of 80 per cent or more of the pigment, a one-third reduction in optical density and no loss in specific biological activity of the effluent. However, the absorption maximum of the effluent remained at 285 mμ. More recently, a procedure combining ethanol precipitation and dialysis for four days against 0·16M-NaCl and 0·025M-PO_4 in 20/32″ Visking casing followed by passage of the dialysand through DEAE at pH 7·4 yielded a highly purified active effluent with an absorption peak at 280 mμ and practically no pigment.

Table II indicates the relative potencies of several thalassaemia urine preparations. Comparisons are made with the results achieved by the four-step procedure employed by White and co-workers (1960) for phenylhydrazinized sheep plasma. It is noted that the purity obtained in the ethanol-precipitated or kaolin-eluted urine materials is considerably greater than that occurring in the Step III procedure of White and co-workers (1960) for sheep plasma. In fact, the kaolin eluate of run 10 approaches that seen in White's Step IV procedure. It would also appear that considerable amounts of carbohydrate may be separated from the urinary preparations without impairment of erythropoietic activity. Indeed, the preparation with the highest specific activity in Table II, the kaolin 10 eluate, exhibited the least carbohydrate (anthrone reaction). These preparations contain considerably less carbohydrate than residues of ultrafiltered urine from patients with aplastic anaemia (Van Dyke, Garcia and Lawrence, 1957*a*, *b*). In view of the low electrophoretic mobility

2 contained 10 mg. of once-crystallized salt-free trypsin in 4 ml. solution. Thirty-four ml. of the urinary preparation at pH 7·4 was brought to 37° by incubation for 30 minutes in a water bath. Two-tenths ml. of the enzyme mixture was added and the incubation terminated at the end of five minutes by the addition of 0·2 ml. soy-bean trypsin inhibitor solution (10 mg. per 2 ml. of solution). A second 34 ml. sample of the material was allowed to remain for 30 minutes in the water bath at 37°, following which a 30-minute incubation with 0·6 ml. trypsin mixture was conducted and then terminated by the addition of 0·4 ml. inhibitor solution. A third 34 ml. portion, after being incubated at 37° for 30 minutes, was digested with 1·2 ml. of the enzyme mixture for 3½ hours, following which 1·0 ml. of inhibitor solution was added.

Assay procedure. The method employed was a modification of that used by Fried and co-workers (1957). Twenty-five adult female rats (wt.—195–225 g.) were divided into five groups. The animals were then deprived of food for the duration of the assay. On the third and fourth days of starvation, the animals of three groups received two daily 2·5 ml. subcutaneous injections of the enzyme incubates. A fourth group was injected on the third and fourth days with 2·5–3·0 ml. of urine preparation incubated at 37° for 3½ hours with no added trypsin. A fifth group served as untreated controls. Six hours after the second injection, and at a similar time in the untreated controls, the rats received an intraperitoneal injection of 1 μc ^{59}Fe per 100 g. body weight. The specific activity of the isotope was 6 mc per mg. iron. Eighteen hours after the radioiron injection, the rats were lightly etherized and the blood drawn by cardiac puncture into heparinized Vacutainer tubes. The packed cell volumes of the blood sample were determined by the microcapillary haematocrit method. After measuring the activity of 1 ml. of whole blood in a well-type scintillation counter, the radioiron uptake could be expressed as counts per minute per ml. of packed cells.

of the 1M-NH_4OH kaolin eluates (Winkert *et al.*, 1958*a*, *b*; Gordon *et al.*, 1959*c*) it seems likely that the alkaline elution procedure has resulted in the separation of considerable electronegative carbohydrate from the urine with no associated loss in biological activity. However, the necessity for some carbohydrate in the molecular structure is seen from the recent finding of Borsook (1959*a*) that incubation of the plasma ESF, from phenylhydrazinized rabbits, with Burnet's neuraminidase for ten minutes at pH 7 resulted in complete loss of erythropoietic activity. This and other findings (Rambach *et al.*, 1958) have suggested that sialic acid is an essential component of the ESF molecule.

Because of the susceptibility of erythropoietically-active urine preparations to trypsin or chymotrypsin (Van Dyke *et al.*, 1957*a*; Gordon *et al.*, 1959*c*), it has been inferred that the urinary ESF is a polypeptide or that a polypeptide portion is essential for its biological activity. A similar conclusion was first arrived at by Niu (1958) for an inducing factor from thymus tissue. It developed subsequently that the presence of proteolytic enzyme in his bioassay system was inhibiting the action of the inducing factor. Addition of equimolar amounts of soy-bean trypsin inhibitor to the trypsin-inducing factor incubates led to the restoration of the activity of the inducing factor. His findings that the factor displayed an absorption maximum at 265 mμ and was destroyed by ribonuclease suggested that the inducing activity was associated with ribonucleic acid (RNA) type structure. The following experiments were conducted to preclude a possible erythropoietic inhibitory action of the trypsin itself.

Enzyme incubations. The ESF used was a dialysed 80 per cent ethanol precipitate of an active urine from a child with thalassaemia major (Hb—4·0 g.). A mixture containing equal volumes of two trypsin preparations was used for the digestion. Preparation 1 consisted of 10 mg. of twice-crystallized trypsin containing 50 per cent $MgSO_4$ (Worthington) in 4 ml. solution. Preparation

operates within the organism. For the ESF, this probably takes the form of a circulating glycoprotein. But an equally important objective is to determine the active "cores" within the larger molecular structure. Although such procedures, generally accomplished enzymically, lead to reduction in biological activity, they are essential in elucidating the nature of the operating effective groupings, which in the ESF may be polypeptide in nature. In recent years, the value of this approach has been seen clearly in the area of the adenohypophysial (Cohen, 1958) and melanophore-stimulating hormones (Hofmann, Yajima and Schwartz, 1959). Cleavage of a large proteinaceous molecule from animal sources into smaller moieties which retain biological activity may remove an immunological barrier that deters use of the factor in other species including man. The real challenge is to reconstruct back, chemically and physically, the maximally active molecule from the dissected cores and carrier substances. It seems likely that chemical work on the ESF will soon take this course.

Further evidence for the lack of species-specificity in the action of the human urinary ESF

Effects in the guinea pig

[illegible] have reported an inability of a urinary pre- [illegible]

to stimulate RBC formation in the guinea pig, [illegible] tiveness of these preparations in rats Since this represents the first mammalian species reported to be refractory to human and rabbit ESF, it was considered important to re-examine this possibility with the use of a highly active urine from a child with thalassaemia major. This urine sample, in 3 ml daily doses given for five days, induced an increase in reticulocytes from 3·0 to 14·7 per cent and haematocrits from 45·9 to 51·8 per cent (i.e.

Table III indicates that incubation for $3\frac{1}{2}$ hours with a larger concentration of trypsin resulted in complete destruction of the urinary ESF. No activity was observed in the recipient rats despite the addition of an excess of trypsin inhibitor just prior to injections of the incubates. The increase in activity noted with the five-minute incubates is not statistically significant when compared to counts of the RBC of the rats injected with the urine material not incubated with trypsin. More recent experiments have indicated that the lack of activity of the urine—trypsin—soy-bean inhibitor incubates cannot be attributed to an inhibitory action, in the recipients, of the injected trypsin or soy-bean

Table III

Effects of trypsin on the activity of human urinary ESF

Incubation period	*Counts/min /ml. packed cells* (mean values ± S E. of mean)
Untreated controls	5,100 ± 1,070
$3\frac{1}{2}$ hr without trypsin	34,200 ± 8,660
5 min + trypsin	55,500 ± 9,000
30 min + trypsin	33,470 ± 3,160
$3\frac{1}{2}$hr. + trypsin	8,500 ± 3,600

inhibitor since (1) simultaneous addition of trypsin and inhibitor to the active urine preparation did not result in any decrease in the erythropoietic activity of the material when tested in the starved rat, and (2) injections of both trypsin and inhibitor (with no urinary material) did not lower the uptake of radioiron by the RBC of the recipient rats These experiments permit us to re-iterate, with greater confidence, the importance of polypeptide groupings for the biological activity of the urinary ESF.

It might not be amiss at this point to indicate that two approaches are generally taken in the elucidation of the chemical structure of a biologically active substance like the ESF. The first objective is to isolate and characterize the homogeneous molecule with high biological activity as it presumably exists and

Table IV

EFFECTS OF COOLEY'S ANAEMIA URINE, HOMOLOGOUS "ANAEMIC" AND NORMAL PLASMA UPON ERYTHROPOIESIS IN GUINEA PIGS (MEAN VALUES ± S E OF MEAN)

Materials injected	Before injections		After 5 injections		After 10 Injections	
	Retics (%)	Haemat. (%)	Retics (%)	Haemat. (%)	Retics. (%)	Haemat. (%)
Cooley's urine	1·0±0·2	43·6±1·3	4·1±1·0 ($P<0·01$)*	42·4±1·6	4·4±0·16 ($P<0·01$)*	46·0±1·5 (N.S)†
Homologous "anaemic" plasma	0·9±0·1	46·9±1·0	6·9±0·39 ($P<0·01$)*	46·4±0 9	—	—
Homologous normal plasma	0·8±0·1	48·9±0·9	1·3±0·16 (N.S)†	44·7±1·3	—	—

* Probability values calculated from the distribution of Fisher's *t* Values after 5 or 10 injections compared to values before injections.
† N S—Not significant

17·6 H-R units) in our standard intact female rat assay. Six adult *male guinea pigs of the Hartley strain received subcutaneous* injections of 15 ml. per kg. of the filtered but otherwise untreated urine daily for periods of five and ten days. For comparison, six guinea pigs were injected daily for five days with 15 ml. per kg. of homologous plasma obtained from thrice-bled donors. Six animals, injected with 15 ml. per kg. of plasma from normal donors, served as controls. Haematocrit and reticulocyte values were used as the erythropoietic parameters.

Table IV indicates that ten injections of the urine specimen employed evoked significant increases in the reticulocyte levels and a tendency towards a rise in haematocrit values. Reticulocytosis was also evident after five injections of the urine. Five injections of homologous "anaemic" plasma induced a somewhat greater reticulocytosis than seen with the urinary material, although here again there was no associated elevation in the haematocrit values. It should be stated that treatment with other urine samples, erythropoietically active and non-toxic in rats, failed to produce a positive response in intact guinea pigs. In some cases, marked decreases in haematocrit values were experienced by the treated animals.

It would appear that the response of the guinea pig to the human ESF is variable and not as pronounced as that displayed by the rat. In this regard, it seems likely that the guinea pig exhibits a greater sensitivity to the non-erythropoietic immunological factors in human urine than does the rat, a phenomenon that would tend to mask its erythropoietic-stimulating properties in the guinea pig. This is in essential agreement with the conclusions of Borsook (1959*b*). The ability to detect erythropoietic-stimulating activity in the guinea pig with some urinary samples may relate to differences in the amounts and types of antigenic material present. It seems important to re-examine the erythropoietic response of the guinea pig to the considerably more highly purified plasma and urine preparations of ESF now available.



Fig 6 *Desmognathus phoca* (× 1)

Effects in *Desmognathus phoca*

Desmognathus phoca (Fig. 6), a salamander indigenous to the mid-South Atlantic regions of the United States, was selected for ascertaining the effectiveness of human urinary ESF upon erythropoiesis in a lower vertebrate because of the ease with which its spleen could be dissected and because of its availability throughout the year from the dealer (Carolina Biological Supply Company, Elon College, North Carolina).

A preliminary feeding period with Tubifex for two weeks served to reduce the variability in response noted with newts started directly upon receipt from the dealer. Sixteen salamanders of both sexes, ranging in weight from 2 to 4 g., were injected pleuroperitoneally with 0·5 μc ^{59}Fe per g. of body weight. Six hours later, the counts per minute per mg. averaged as follows: *head*—56 4; *tail*—59·6; *liver*—219·0; and *spleen*—546·0, a finding consistent with the view that the spleen is the primary site of erythropoiesis in urodeles (Jordan, 1938).

Another group of 12 salamanders was injected daily on three successive mornings with 0·2 ml. of a highly active dialysed ethanol precipitate of urine from a child with thalassaemia major. Six hours after the last injection, 0·5 μc of ^{59}Fe was injected pleuroperitoneally per g. of body weight. The uptake was ascertained similarly in 12 controls not receiving the urinary extract. The animals were killed at various times for determination of splenic radioactivity. In several animals, determinations were also made of the incorporation of the radioiron into the circulating RBC at 54 hours after isotope injection

Fig 7 indicates that the uptake of ^{59}Fe was greater in the spleens of the ESF-injected salamanders than in the controls at the three periods examined. A maximum uptake was attained at 36 hours which was maintained at the 54-hour period. By 54 hours, the incorporation of the isotope into the circulating RBC of the ESF-treated animals was approximately 120 per cent greater than



Fig 6 *Desmognathus phoca* (× 1)

in the controls (i.e. 30,050 counts per minute per optical density unit * in the experimental animals as compared to 13,400 in the controls). At the 54-hour period, a comparison showed approximately 2,400 counts per minute per mg. spleen and 960 counts

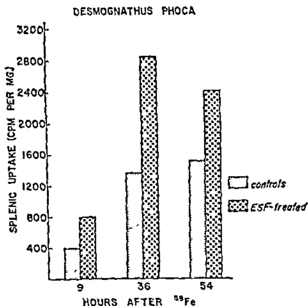


FIG. 7 Splenic uptake of ^{59}Fe (counts per minute per mg wet spleen) in control and human urinary ESF-treated salamanders

per minute per mg of whole blood. Microhaematocrits established that the circulating RBC accounted for approximately 98 per cent of the activity in blood at this time. These preliminary experiments appear to indicate that the ESF of human origin stimulates erythropoiesis in the urodele.

* Determined by haemolysing the blood sample, obtained at the site of decapitation, in 3 ml of distilled water for two hours, spinning down the sediment and reading the supernatant at 540 mμ in the spectrophotometer.

Evidence for a circulating leucocytosis-inducing factor (LIF)

Experiments have been described supporting the existence of a circulating LIF (Gordon, 1959*b*; Gordon *et al.*, 1959*a*). The evidence is derived from (1) the leucocyte behaviour in parabiosed rats, one partner of which is subjected to repeated leucocytapheresis (LAP), (2) studies on the effects of "LAP plasma" on the leucocyte picture in intact rats, and (3) observations on the influence of "LAP plasma" on leucocyte discharge from isolated perfused hind legs of rats. Some of these studies have recently been extended and a summary of the essential findings appears below. Full details are provided in the paper by Gordon and co-workers (1960).

Parabiotic experiments

Adult female rats of the Charles–River strain weighing 180–200 g. were used. Parabiosis was performed between littermates by the method of Bunster and Meyer (1933). Abdominal cavity LAP was initiated four to seven days after parabiotic surgery. Twenty-five ml. of non-pyrogenic isotonic saline was injected intraperitoneally into the right parabiont of each pair. Nine hours later, when most of the injected fluid had been absorbed from the cavity, an additional 10 ml of saline were injected intraperitoneally for the purpose of flushing the cavity and collecting the accumulated leucocytes. This LAP procedure was repeated several times, usually six to eight, until at least one thousand million cells had been removed from the right partners. Repeated LAP resulted in the appearance of progressively greater numbers of polymorphonuclear leucocytes in the abdominal exudates. Although the *percentages* of mononuclear forms (lymphocytes and macrophages) tended to diminish, the *absolute* numbers of the mononuclear elements increased with each withdrawal because of the greater total numbers of cells appearing in the exudates.

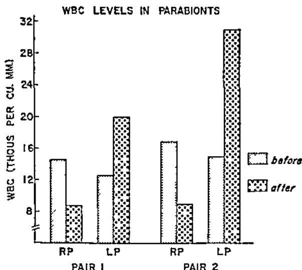


FIG 8. Total peripheral leucocyte counts in parabionts before and after repeated LAP in right partners RP—right partner, LP=left partner

NEUTROPHIL AND LYMPHOCYTE LEVELS IN PARABIONTS

NEUTS (THOUS PER CU. MM)

LYMPHS (THOUS PER CU. MM.)

RP LP RP LP
PAIR 1 PAIR 2

before after

FIG 9. Peripheral neutrophil and lymphocyte counts in parabionts before (B) and after (A) repeated LAP in right partners RP=right partner; LP=left partner

Leucocyte determinations were made from either freely-flowing tail blood or from cardiac blood in both parabionts before initiation of leucocyte removal from the right partner and at four to six hours after the last LAP had been performed. The peripheral white cell changes for two representative pairs, of a total of ten pairs examined, are depicted in Figs. 8 and 9. Repeated LAP performed in the right partners generally resulted in a significant drop in their peripheral white cell counts. With one exception, this was accompanied by a significant rise in the white cell counts of the left non-LAP partners. The leucocyte increases in these latter rats were due to rises in lymphocytes, polymorphonuclear neutrophils and eosinophils (Fig. 9).

Plasma injections

"LAP plasma." The LAP procedure used in the parabiotic series was performed in 25 intact rats which served as the plasma donors. Approximately 1,500 million leucocytes were removed from each of the donors. Each of six normal adult female rats received a single 10 ml. subcutaneous injection of this "LAP plasma"; another six rats were given single injections of 10 ml. of plasma from non-LAP animals. Table V indicates that the "LAP plasma" evoked, at four and six hours, highly significant increases in the white cell counts of the intact recipient rats. These increases were largely the result of rises in the numbers of neutrophilic granulocytic and lymphocytic elements. There was, however, a greater relative increase in the neutrophil numbers which increased percentage-wise while the lymphocyte percentages decreased somewhat. Control plasma was ineffective on any of the parameters studied.

Additional controls. The importance of the removal of the leucocytes in the production of the LIF was pursued in 15 rats subjected to sham-LAP, i.e. procedures were followed similar to those indicated above except that the exudates and their contained leucocytes were not removed. Single 10 ml. injections of plasma

Table V

EFFECTS OF LAP AND NON-LAP PLASMA UPON PERIPHERAL WHITE CELLS OF INTACT RATS (MEAN VALUES ± S E OF MEAN)

Source of plasma	*No of recip rats*	*Hr after inject*	*WBC (thous / cu mm)*	*Eos. no / cu mm.*	*Differentials (%)*	
					Mononucl	*Neuts*
LAP rats	6	0	15·6±2·9	183±34·6	85±1·8	15·7±2·2
		4	37·5±4·5 (P<0·01)*	296±3·7 (P<0·01)*	72±3·9 (P<0·01)*	27·7±3·9 (P<0·01)*
		6	34·0±3·2 (P<0·01)*	267±34·5 (N S)†	72±3·0 (P<0·01)*	26·7±2·9 (P<0·01)*
Normal (non-LAP) rats	6	0	16·8±2·5	285± 85·9	86±1·4	13·3±1·1
		4	17·8±2·4	318±105·8	86±2·5	13·8±2·2
		6	17·4±2·4	313±104·1	83±2·2	15·7±2·1

* Probability values calculated from the distribution of Fisher's *t*. Values at 4 or 6 hours compared to values at 0 hours.
† N S—Not significant

Leucocyte determinations were made from either freely-flowing tail blood or from cardiac blood in both parabionts before initiation of leucocyte removal from the right partner and at four to six hours after the last LAP had been performed. The peripheral white cell changes for two representative pairs, of a total of ten pairs examined, are depicted in Figs. 8 and 9. Repeated LAP performed in the right partners generally resulted in a significant drop in their peripheral white cell counts. With one exception, this was accompanied by a significant rise in the white cell counts of the left non-LAP partners. The leucocyte increases in these latter rats were due to rises in lymphocytes, polymorphonuclear neutrophils and eosinophils (Fig. 9).

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in the total numbers of circulating white cells at the six-hour period.

Action of the LIF on the bone marrow. Single 10 ml. injections of the "LAP plasma" caused, at six hours after administration, significant decreases in the absolute numbers of neutrophilic and eosinophilic granulocytes, accompanied by an increase in the numbers of lymphocytes within the tibial bone marrows of the recipients (Fig. 10). Control plasma also tended to produce somewhat lower neutrophilic cellular and higher lymphocytic numbers but the effects were not significant. Nucleated RBC as well as total nucleated cell numbers were not affected by either normal or "LAP plasma".

Direct actions of the LIF

The effects of "LAP plasma" as well as normal plasma were studied on the rate of leucocyte release from isolated hind legs of rats perfused with 50 ml. of homologous blood depleted of most of its leucocytes by repeated centrifugation and removal of the buffy coats. The details of the perfusion system have been described previously (Gordon *et al*, 1959*c*; Kuna *et al.*, 1959). Four groups were utilized. The first group (five legs), which served as controls, established the rate of leucocyte release under the standard conditions of the perfusion system. In the second group (three legs), 7 ml. of "LAP plasma" were added to the perfusate at the beginning of the perfusion period In the third group (three legs), 7 ml. of plasma from non-LAP rats were added at the start and 6–7 ml. of the "LAP plasma" added at 30 minutes. Lastly, to two perfused preparations, 25 µg. endotoxin (Difco's Staphylococcus A lipopolysaccharide) were added at the beginning and another 25 µg. at one hour. All perfusions were conducted over a two-hour period.

Fig. 11 indicates that perfusion of isolated hind legs of rats is accompanied by a continuous release of leucocytes into the perfusate. Approximately 50 million are released in 30 minutes.

from these donors into each of five rats produced no significant alterations in white cell numbers or differentials at the four and six-hour periods.

The leucocytosis-inducing action of the "LAP plasma" is probably not attributable to non-specific factors that might be implicated in the response to the stress of the leucocyte removal

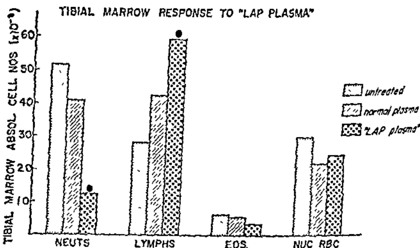


FIG. 10. Mean absolute numbers [illegible] of rats at 6 [illegible]

procedures. Thus the intensity of the absolute increases in peripheral white cell counts as well as the temporal features of the response evoked by the LIF were not reproduced by single injections of prednisolone (1 mg.), Adrenalin (Parke, Davis & Co., 10 µg.), L-arterenol (noradrenaline) (200 µg.), serotonin (25 µg.), histamine (10 µg.) and pyrogen (Baxter Labs., Pyromen, 10 and 100 µg.). The effects generally induced by these agents were characteristic of non-specific stress, namely absolute lymphopenia, eosinopenia and neutrophilia, with no significant changes

is noted that 7 ml. of normal plasma added at the commencement of the perfusion did not significantly alter the rate of leucocyte release during the first half-hour. However, addition of 6–7 ml. of the "LAP plasma" at the 30-minute period induced a significant increase in the numbers released over the next 15 minutes

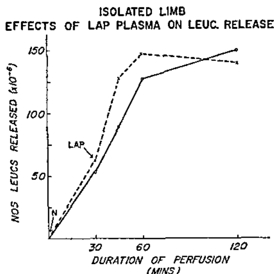


FIG 12 Effects of a single addition of 7 ml of normal plasma at the initiation of perfusion and 6–7 ml of "LAP plasma" at the 30-minute period of perfusion [illegible]

when compared to the same interval in the controls Not noted in Fig. 12 is the finding that two additions of endotoxin to the perfusate did not change the rate of leucocyte release from the perfused preparations.

The experiments cited above favour the existence of a circulating LIF following withdrawal of large numbers of leucocytes

At one hour, the number has more than doubled and values of approximately 150 million are attained by two hours. Neutrophilic granulocytes and lymphocytes constitute the greater majority of the cells released, with the former outnumbering the latter. That the leucocytes are derived primarily from the bone marrow is seen from the fact that demedullation of the long bones

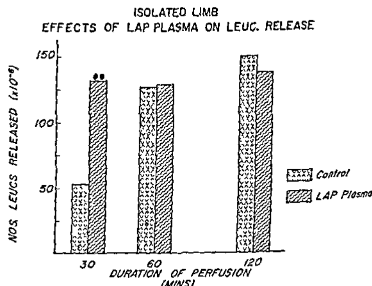


FIG. 11 Effects of a single addition of 7 ml. of "LAP plasma" at the initiation of perfusion on the rate of leucocyte release from isolated hind legs of rats perfused with leucocyte-depleted blood.

of the legs prior to perfusion markedly reduces the numbers of leucocytes released into the circulation.

Addition of 7 ml. of "LAP plasma" at the initiation of perfusion resulted in a 150 per cent increase in the numbers of leucocytes released over that observed in the control preparations. However, by one and two hours after addition of the "LAP plasma", the numbers of leucocytes counted in the perfusates were no greater than those noted at 30 minutes. From Fig. 12 it

Komiya (1956) has called attention to a variety of "leukopoietins" in the blood of rabbits within a few hours after injection of typhoid vaccine, pertussis vaccine and other agents. Drs. Komiya and Katsunuma of the Tokyo Medical College Hospital have kindly provided us with a sample of their purified rabbit plasma, "neutropoietin", evoked by typhoid vaccine, which appears to be polypeptide in nature (Komiya *et al.*, 1959) and has the same migration velocity as a γ-globulin in the paper electrophoresis system (Komiya, personal communication). In our hands, the material has proved active in causing a marked peripheral neutrophilia in rats at four hours after single intravenous injections of 250 μg. We have also recently detected the appearance of a leucocytosis-inducing activity in the plasma of rats at four to six hours after subcutaneous injection of 0 5 ml. of typhoid-paratyphoid vaccine (New York City Health Department). The peripheral leucocytic pattern produced by a single 10 ml. injection of this plasma in rats is quite similar to that induced with the "LAP plasma". It is possible that the typhoid vaccine operates by causing the emigration of leucocytes to sites where their inhibitory action is not operative or through their eventual destruction. This would provide a unitary hypothesis for the appearance of an LIF in the plasma of LAP and vaccine-injected animals

Summary

Studies are reported on the erythropoietic-stimulating factor (ESF) in human and sheep urine A positive correlation exists between the intensity of anaemia in children with thalassaemia major and the presence of the ESF in their urines. The factor disappears promptly following transfusion Strong erythropoietic-stimulating activity is also detectable in the urines of sheep rendered intensely anaemic with phenylhydrazine.

Normality of the RBC evoked in rats in response to the human urinary ESF is indicated from studies of their osmotic fragility,

from rats. The action of the LIF appears to be exerted directly on the bone marrow, causing a rapid release of mature granulocytes and lymphocytes into the circulation. One can only speculate regarding the mechanisms responsible for the appearance of the LIF following repeated LAP. The concept has been advanced that the adult non-proliferative members of a cell lineage act to inhibit the proliferation of the stem elements of that series (Weiss, 1954; Osgood, 1955; Rose, 1955). A similar negative feedback could conceivably operate for the blood cell release phenomenon in which withdrawal or destruction of adult leucocytes, with the removal of their contained inhibitory mechanism, would spark the production of the humoral LIF. Evidence for this concept is provided in the present findings that when the peritoneal exudates and their contained leucocytes are not removed following repeated injections of saline into rats, their plasma possesses no leucocytosis-inducing activity. It is also possible that the release of leucocytes from the bone marrow, provoked by the LIF, triggers a compensatory cellular proliferative activity to replace the elements discharged into the circulation. This would be analogous to the situation for the RBC in which the earliest morphological sign of enhanced erythropoietic activity is a peripheral reticulocytosis (Seip, 1953), signalling a subsequent increased proliferation of the erythroid precursors in the bone marrow.

Another explanation envisages an inherent ability of the adult leucocytes to metabolize a normally circulating LIF. Withdrawal, immobilization or destruction of leucocytes would permit the accumulation and action of this factor. This hypothesis is being tested by determining whether incubation of "LAP plasma" with leucocytes results in a diminution of its activity.

The LAP-induced LIF remains to be purified and characterized. Preliminary studies reveal it to be heat-labile and resistant to freezing. In addition, "LAP plasma" shows an accentuation of the γ-globulin band in the paper electrophoretic system.

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oxygen content and capacity as well as the alkali denaturation and electrophoretic mobility of their contained haemoglobin.

Kaolin-adsorption and ethanol precipitation methods are described for the purification of the human urinary ESF. Present indications are that the factor is a mucoprotein with associated polypeptide groupings important for the biological activity.

The response of the guinea pig to the human urinary ESF is of lower order of magnitude than that displayed by rats. Increased uptake of radioiron by the spleens and augmented incorporation of the isotope into the circulating RBC occur in the salamander, *Desmognathus phoca*, following injections of the human urinary ESF.

Further evidence is presented supporting the existence of a leucocytosis-inducing factor in the plasma of rats subjected to repeated leucocytapheresis. It appears to operate by increasing the rate of leucocyte release by a direct action upon the bone marrow.

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I wish to express my sincere thanks to the following for their active collaboration in various phases of the experimental work reported in this paper. Drs. B. S. Dornfest, M Eisler, C D. Siegel and J W. Winkert, Messrs E. Handler, J LoBue, R Neri, A Weintraub, and Mrs Leslie Jacobson.

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Roylance: S. Osnes (1958. *Brit. med. J.*, **2**, 1387) did some histological work on this.

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Gordon: The effects reported originally by Prof. Yoffey and his group (Harris, P. F., Menkin, V , and Yoffey, J M. [1956] *Blood*, **11**, 243) were obtained with Menkin's leucocytosis-promoting factor derived from a turpentine-induced inflammatory exudate in dogs. I have no idea whether there is any chemical similarity between Menkin's material, which is associated with the α-globulins, and ours, which appears to be linked to the γ-globulin fraction of plasma. There is, however, a similarity in the action induced by the two factors on the cellular elements of the bone marrow.

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DISCUSSION

Roylance Dr. Gordon, you mentioned that many workers had tried to elucidate the erythropoietic-stimulating factor from various organs. Have you done any work yourself on the site of manufacture?

Gordon We performed some experiments (Gordon, A S., Piliero, S J, and Tannenbaum, M. [1955]. *Amer. J Physiol.*, 181, 585) in which rabbits were subjected to repeated bleedings These experiments were repeated using phenylhydrazine to induce anaemia (Piliero, S. J., Medici, P. T, and Gordon, A. S., unpublished). Erythropoietically-active extracts were obtained from the plasma of these animals by the acidification-boiling procedure used by Borsook. We reasoned that since these procedures uncovered activity in plasma, they should be capable of revealing erythropoietin in those organ systems concerned with its production However, we were unsuccessful in detecting impressive activity in filtrates of liver, packed red cells, skeletal muscle, brain, pancreas, kidney, lung, skin, thymus and intestinal tract. These

experiments were performed before the provocative findings by Jacobson and co-workers were reported, on the kidney as a site of production. Recently, Eugene Goldwasser presented some preliminary evidence for erythropoietic-stimulating activity in homogenates of kidney from anaemic rabbits (1959. Erythropoietin Symposium. Sponsored by the Atomic Energy Commission, Washington, D.C.). Similar findings have been reported by J. P. Naets, for homogenates of kidney from anaemic dogs (1960. *Proc. Soc. exp. Biol.* (*N.Y.*), **103**, 129). Our negative results with kidney might have been due to a destructive effect of the acidification-boiling procedure on the factor in the extracts.

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Osmond: I wonder if this is in fact a specific response—it is a thing we see in response to many stimuli.

Gordon: Nevertheless this does not preclude the possibility that it constitutes a normal physiological reaction accompanying the enhanced release of leucocytes from the bone marrow. The response of the guinea pig to erythropoietin appears to be a variable one. We have tested in the guinea pig several specimens of urine from thalassaemia major subjects which were highly active in rats. The urines generally induced haemolytic effects as evidenced from the significant decline in haematocrit accompanying the reticulocytosis. With one urine sample, however, we obtained evidence of a true stimulation of erythropoiesis (Table IV, p. 347) This may have been due to the fortuitous absence, in this urine, of antigenic factors to which the guinea pig is more responsive than the rat. Apart from immune reactions, the rat appears to respond much more sensitively to erythropoietin than does the guinea pig.

Van Dyke: Dr. G. Keighley has shown that antibody formation may destroy the effectiveness of erythropoietically active rabbit plasma in rats when injected for several days, but not during the time required for the ^{59}Fe red cell incorporation assay. We found absence of response in the guinea pig to human urinary erythropoietin, using the ^{59}Fe assay, where one would not expect that there had been sufficient time for antibody production to get under way.

You mentioned privately that in your method of collection of human urinary erythropoietin the time of exposure to the kaolin was important in recovering the activity. That could be a pitfall for someone who was trying the method for the first time.

Gordon. The kaolin procedure may yield variable results. In several runs we obtained no recovery of activity with the kaolin adsorption technique. One point of importance is to allow the urine to remain in contact with the kaolin for only a short time. Exposure for too long a time may result in varying degrees of denaturation with accompanying loss of biological activity. Although the degree of purification achieved with the kaolin technique may be high, the yields obtained have not generally been as consistent as with the ethanol precipitation-prolonged dialysis procedure, at least in our hands. We have now resorted almost completely to the latter method for obtaining reason-

ably purified material from active urine. A good deal of the pink pigment associated with these preparations may be removed by passing

rabbit plasma.

Lamerton: Have you perfused tissues or organs other than the hind limb with the leucocytosis-inducing factor? Do you get leucocytes out of lung and liver, for instance? Such experiments might throw some light on the extent of the extramedullary stores of the leucocytes.

Gordon: We have not yet attempted to do this with other organs. With regard to erythropoietin, however, Dr. S. Kuna, Mr K Melman and I are now perfusing isolated rat kidneys and we intend to join a leg to the circuit By reducing the oxygen content of the blood perfusing through the kidney we hope to induce it to produce erythropoietin which may then stimulate erythropoiesis in the marrow of the leg.

Jacobson: Some discussion on this problem of non-specificity might be worth while I assume from what has been said by Dr. Lajtha and Dr. Gordon that the stimulation of the stem cell is specific for erythropoietin. But non-specific things could certainly affect the haemoglobin content of the erythroblast and its later stages and therefore one has to be very careful to consider this from the point of view of separating the two aspects of the problem.

Have you tried erythropoietin on lower forms in which there were no red cells and haemoglobin was soluble?

Gordon. Several years ago I made an attempt to use *Daphnia* as an assay animal for erythropoietin, since this form possesses a measurable

find a vein to inject, so we added the plasma erythropoietin preparations to water containing large numbers of *Daphnia pulex* Unfortunately, we could find no evidence that haemoglobin synthesis in this form was enhanced by the factor.

With regard to the matter of non-specificity, it must be emphasized

that an increase in red cell mass in the *intact animal* can be induced only by oxygen deficiency, in one form or another, or by the mediator of anoxia, namely erythropoietin. This is why we prefer to use the intact animal for our assays of erythropoietin.

Stohlman: How do you account for compensated haemolytic syndromes?

Gordon: I am willing to concede that there may be additional mechanisms involved in the regulation of erythropoiesis as there are in the control of leucopoiesis. Again with respect to the matter of erythropoietin assay, the intact animal has the advantage of reacting in a more specific manner to erythropoietin. Factors such as folic acid, liver and vitamin B_{12} (Hodgson, G., and Tohá, J [1954]. *Blood*, 9, 299), as well as other vitamins, the intrinsic factor, iron and copper (Wintrobe, M. M. [1956]. Clinical Hematology, 4th edn. Philadelphia· Lea), although dramatically effective in animals lacking these principles, exercise little or no action on erythropoietic processes in intact animals subsisting on a normal diet and possessing a normal responsive marrow.

Craddock: In your perfused hind limb preparation are the bulk of the cells that come out on perfusion the same as you describe in your intact animal blood differential—in other words do both lymphocytes and granulocytes come out?

Gordon: Yes, both types of cells are discharged and the release of these elements into the perfusate is accompanied by a partial depletion of the granulocytic population in the perfused marrows. Lymphocyte numbers in the marrows may not necessarily change, indicating that those which are released are being replaced by some as yet imperfectly understood mechanism. It is to be emphasized that in the leucocytapheresis procedure employed by us, both granulocytic and mononuclear elements are being removed. Before leucocytapheresis, the peritoneal fluid of the rat contains largely mononuclear elements, some eosinophils and mast cells. With repeated leucocytapheresis, the picture changes. Polymorphonuclear neutrophils in increasing numbers infiltrate the exudate. However, large numbers of mononuclear cells persist in the fluid. If we consider the possibility that each adult cell type feeds back negatively to its own precursor element, the effects of the leucocytapheresis plasma on both granulocytes and mononuclear

cells become more understandable We intend to test the validity of this hypothesis by determining whether the plasma of rats, from which large numbers of lymphocytes have been removed through thoracic duct cannulation, possesses specific lymphocytosis-inducing properties. It is perhaps too much to expect that it will be active but it seemed to us that this might be an appropriate way to test the validity of the specific cell type, negative feedback humoral hypothesis.

Craddock: When you perfuse the limb with normal cell-free, lymphocyte-free, plasma, is the discharge of cells under those conditions more than if you perfuse with blood containing a normal sample of white cells?

Gordon: If isolated legs are perfused with blood containing normal numbers of leucocytes, the absolute numbers of elements entering the perfusate from the marrow are less than when leucocyte-free blood is used as the perfusate. Whether this is a humorally-mediated or a mechanical effect, we are not in a position to state. We hope to test the humoral possibility by addition of leucocyte homogenates and extracts to the perfusate.

Braunsteiner: How do you inject the 10 ml. of plasma?

Gordon: Subcutaneously in two 5 ml. doses, one behind the neck and the other just above the tail. Incidentally, single 5 ml injections are ineffective; 10 ml. seems to be the minimum effective dose.

Rachmilewitz: You have shown very impressive evidence for the existence of the LIF produced by leucocytapheresis. In conditions associated with increased erythropoietin production, such as bleeding or haemolysis, there is the same effect of leucocytosis, which does not support the existence of a separate factor. How do you explain this leucocyte response to erythropoietin in conditions associated with increased erythropoietin production?

Gordon: In some of our early experiments, we administered 18 to 24 injections of an active plasma erythropoietin preparation and we saw no dent in the leucocyte picture. On the other hand, the leucocytosis-inducing factor does not appear to produce any change in the peripheral red cell numbers or in the numbers of nucleated red cells in the bone marrow We intend to inject rats continuously for very long periods with purified urinary erythropoietin. Perhaps some change in the leucocyte picture will eventually occur. Is it possible that the

animal eventually adapts to the erythropoietic-stimulating factor and converts it, through a minor change in its chemical structure, into a leucopoietic factor? It is not beyond the realm of possibility that a variety of "poietins" exist possessing the same basic structure, with differences in prosthetic groups that determine whether it will stimulate granulocyte, lymphocyte or red cell production.

Rachmilewitz: Yet in the animal experiment the leucocytosis is a very acute effect following bleeding or haemolysis, which I thought were conditions associated with increased erythropoietin production.

Jacobson: You are saying that the act of bleeding the animal has an effect on leucocytosis, not that it is an effect of erythropoietin.

Gordon: I must have misinterpreted the question.

FACTORS IN THE CONTROL OF HAEMOPOIESIS*

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THE problem of the physiological and pathophysiological control of haemopoiesis is complex and as yet unsolved in spite of recent advances in our knowledge of the kinetics of blood cell production Many questions remain unanswered. However, the existence and fundamental importance of a humoral erythropoietic regulatory mechanism has now received unequivocal confirmation by numerous investigators. Experimental data also support the thesis that the formation of other haemic elements may be subject to similar controlling factors.

Observations in our laboratories on plasmas obtained under a variety of clinical and experimental conditions indicate that there are two humoral erythropoietic factors with different chemical and physiological attributes (Linman and Bethell, 1960). The erythrocytic responses in normal rats given unmodified active plasmas is characterized by an increase in all of the usual parameters which reflect altered erythropoietic activity. In addition, the enhancing effect exerted by these test materials on haemoglobin synthesis is detectable by other techniques such as the incorporation of ^{59}Fe in haemoglobin. The filtrates of plasmas which have been processed by boiling for five minutes or less evoke similar responses in recipient animals, but plasmas subjected

* These studies were supported in part by research grants from the National Institutes of Health, United States Public Health Service

† Deceased.

to more prolonged boiling fail to augment ^{59}Fe uptake, haemoglobin levels, or circulating red cell mass (Linman, Korst and

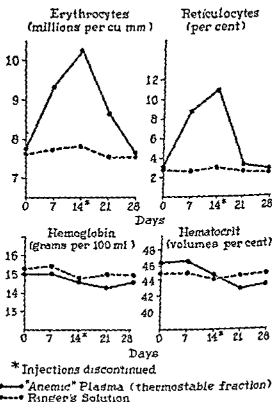


FIG. 1. Erythrocytosis and reticulocytosis without associated change in haemoglobins or haematocrits in normal rats given the thermostable fraction of phenylhydrazine-induced anaemic rabbit plasma. Daily subcutaneous injections were equivalent to 2 ml. of the original plasma per 100 g. of each recipient's body weight. Average determinations of 12 rats in each group.

Bethell, 1959). The relative thermolability of the humoral factor which stimulates haemoglobin synthesis has also been emphasized by others (Stohlman and Brecher, 1957; Gurney and

Pan, 1960). Although such heat-denatured materials are seemingly devoid of erythropoietic activity when studied by techniques employing changes in haemoglobin production as a measure of response, the thermostable fractions of active plasmas are still capable of exerting a profound effect on erythropoiesis.

Normal rats given multiple daily injections of the extracts of active plasmas, which have been boiled over a direct flame for 30

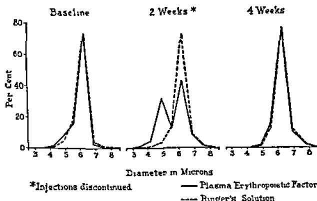


FIG. 2 Graphic demonstration of the microcytes responsible for the erythrocytosis induced in normal rats by the thermostable portion of anaemic rabbit plasma Composite red cell diameter distributions (Price-Jones) of six rats in each group Two weeks after the injections were stopped, the small cells were no longer discernible. (From Linman and Long, 1958 Reproduced from *Blood* by permission)

minutes or more prior to testing, manifest a singular type of erythropoietic response. These animals develop erythrocytosis and reticulocytosis without associated change in their haemoglobin or haematocrit values (Fig. 1) The small cells responsible for the erythrocytosis are readily apparent on stained films and easily demonstrable by Price-Jones measurements (Fig. 2). Recipients of such test materials also exhibit increased marrow nucleated red cell counts (Fig 3) which provide, together with the changes in the peripheral blood, conclusive proof of enhanced

proliferative activity of myeloid erythrocytic precursors. The only alternative explanation for this unique combination of findings would appear to be erythrocytic cytoclasis with impaired viability of the resultant fragments. However, such a "compensated" haemolytic state, i.e. active haemolysis in the

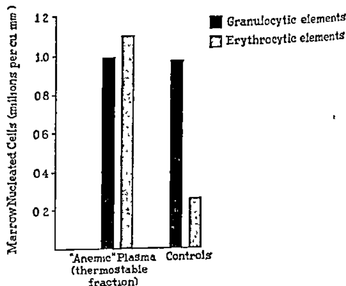
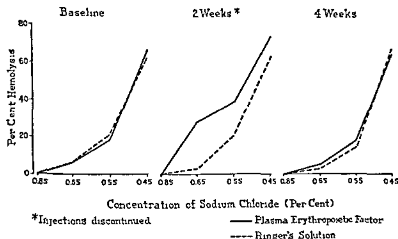


FIG. 3 Myeloid erythrocytic hyperplasia at the end of a two-week injection period in the recipients of the "anaemic" plasma extract which evoked the erythrocytosis and reticulocytosis depicted in Fig 1 Average marrow nucleated cell counts of six rats receiving the plasma extract and a comparable number injected with Ringer's solution.

presence of stable haemoglobin and haematocrit levels, would of necessity be accompanied by an increased rate of haemoglobin production. The latter would enhance ^{59}Fe erythrocytic uptake. Therefore, existent data support the conclusion that the thermostable fractions of active plasmas act as primary erythropoietic stimulants. The ether-soluble fractions of erythropoietically active plasmas induce identical responses in recipient animals

(Linman, Bethell and Long, 1958*a*), whereas the enhancing effect on ^{59}Fe incorporation is retained in the ether-insoluble portions.

The prompt restoration of normal erythroid values after such plasma extract injections are stopped (Fig. 1) coincides with the



significant differences between the experimental and control animals The

by permission)

disappearance of the microcytes (Fig. 2). Osmotic fragility measurements indicate that this phenomenon apparently reflects the impaired viability of the small cells produced in response to this particular stimulus (Linman and Long, 1958). Abnormalities in erythrocyte osmotic behaviour are not detectable by quantitative photocolorimetric techniques in recipients of the

thermostable or ether-soluble fractions of erythropoietically active plasmas. However, decreased erythrocytic resistance to lysis in hypotonic media is easily demonstrable with a direct cell enumeration technique employing red cell pipettes and hypotonic salt solutions as diluents in normal rats given such test materials (Fig. 4). The failure to detect increased fragility by the former method and the simultaneous re-establishment of normal erythrocyte counts, Price-Jones measurements, and fragility curves (Figs. 1, 2, and 4), indicate that, under the conditions of these experiments, only the microcytes possess decreased osmotic resistance.

It has been concluded from these experimental observations that the humoral erythropoietic regulatory mechanism comprises at least two factors with different chemical, physical and physiological properties. The probable modes of action of these humoral agents must be clarified before their rôle in the fundamental control of erythropoiesis may be precisely defined. The erythropoietic activity of certain plasmas and sera has been assumed by many workers to be due to only one substance which stimulates haemoglobin synthesis. However, it is difficult to envisage a single agent exerting basic regulatory control over each of the physiological processes which together result in the formation of the haemoglobin-containing erythron.

The active proliferation and maturation of this highly differentiated series of myeloid cells, together with the acquisition of their cytoplasmic constituents and enzyme systems, requires a carefully regulated chemical system. Nucleoproteins, globin, lipids, porphyrins, and the numerous other essential components are needed in adequate amounts in addition to an intact myeloid reticulum. Subject to its remarkable homeostatic controlling mechanism, erythropoiesis then proceeds in an orderly manner but can be divided into the following four distinct phases: (1) Differentiation of the pluripotential myeloid reticulum cells into red cell precursors, (2) Intramedullary multiplication of nucleated

erythrocytic elements; (3) Maturation of erythrocytic precursors; (4) Synthesis of haemoglobin.

Since the end-product of erythropoiesis is ordinarily confined to the circulation, alterations in erythropoietic activity are readily detectable. However, the type or site of the effect exerted by substances capable of inducing such measurable changes in the peripheral blood can only be inferred from the observable response. For example, demonstrations of enhanced ^{59}Fe incorporation or increases in circulating haemoglobin or red cell mass, although indicative of augmented haemoglobin production, do not define the manner by which it was accomplished.

A number of experimental models have been devised in attempts to elucidate the intricacies of erythropoiesis, but our knowledge is still incomplete. However, it may be reasonably concluded that the complex physiological processes which culminate in the formation of the haemoglobin molecule have their onset in the rubriblast (or proerythroblast) and are inseparably related to the maturation of myeloid erythrocytic precursors. Current data indicate that the intramedullary generation time of the erythrocyte is fixed and predetermined, perhaps by nutritional or hormonal environment. The time required for the development of a mature erythrocyte is apparently independent of the total output by the marrow or the humoral erythropoietic stimulus (Alpen and Cranmore, 1959). Yet, the contribution of erythropoietic tissue to the peripheral blood is clearly subject to both quantitative and qualitative modifications.

Erslev (1959) has proposed that the humoral mechanism initiates erythropoiesis but is not involved in the subsequent maturation or multiplication of erythrocytic precursors. It is readily apparent that an increase in the number of rubriblasts derived from the primitive pluripotential dividing cells will lead to the production of increased amounts of haemoglobin. All available data support the thesis that the relatively thermolabile, ether-insoluble plasma erythropoietic factor, which enhances

haemoglobin synthesis in recipient animals, exerts such an effect. Thus, the oxygen-carrying capacity or haemoglobin content of the blood is most likely determined by the rate of rubriblastic differentiation of the multipotential myeloid reticulum cells. It is reasonable to conjecture that each rubriblast possesses the inherent capacity to synthesize haemoglobin. If all other conditions are normal, including a uniform rate of cellular division, it is equally logical to assume that each such cell is the progenitor of a pre-ordained amount of haemoglobin evenly divided among a given number of red cells.

In contradistinction to the evidence which suggests that humoral factors do not hasten the maturation of erythrocytic precursors, there is no compelling support for the contention that the proliferative activity of nucleated red cells is fixed or that mitotic division, which is basically nuclear, and the synthesis of haemoglobin, largely a cytoplasmic function, must be responsive to a single stimulus. Examples of disparity between these two aspects of erythropoiesis are numerous. They include, among others, the lack of parallelism between erythrocyte and haemoglobin production in hereditary leptocytosis or iron deficiency anaemias and the increased haemoglobin content of the macrocytes in pernicious anaemia *In vitro* bone marrow culture techniques have also shown that the maturation and multiplication of erythrocytic precursors are dissociable and distinct physiological processes.

Increased erythroblastic cellular division would be expected to yield a greater number of cells even in the absence of a comparable stimulus to haemoglobin synthesis, i e. augmented reticulum cell erythrocytic differentiation. However, it may be logically surmised that the new erythrocytes produced under these conditions would be small and contain, collectively, the same amount of haemoglobin as the normal-sized cells which would have been formed from the erythrocytic precursors involved had fewer cellular divisions occurred. Although the duration of the mitotic process is undoubtedly constant, a decrease in the intermitotic

interval or resting stage would permit more mitoses in a given period of time. The unique erythrocytic response observed in normal rats injected with the thermostable or ether-soluble fractions of erythropoietically active plasmas is compatible with and explainable by such an effect

Thus, humoral factors appear to exert both quantitative and qualitative regulatory control over erythropoiesis. A relatively thermolabile, ether-insoluble agent enhances haemoglobin synthesis *most probably by stimulating the pluripotential myeloid* reticulum cells to differentiate into erythrocytic precursors. The number of cellular divisions which the latter undergo during a relatively constant generation time is apparently governed by a thermostable, ether-soluble factor It is suggested that these two humoral factors control, respectively, the quantity of haemoglobin and number of erythrocytes produced and that their combined activities determine, in addition, the size and haemoglobin content of each erythrocyte.

Erythropoietic effects attributable to each of these humoral factors are demonstrable in the plasmas of a variety of experimental animals following acute or chronic haemorrhage, the administration of phenylhydrazine or cobalt, or exposure to lowered barometric pressures. Plasmas and plasma extracts from human donors with certain types of anaemia or with secondary polycythaemia due to arterial hypoxaemia contain similar separable erythropoietic effects. Since microcytes with abnormal osmotic behaviour are not evident *in vivo* in the above donors or in recipients of both erythropoietic factors from exogenous sources, the presence of such cells in normal rats given the thermostable, ether-soluble factor cannot be ascribed to a specific property of this humoral agent. Instead, the small cells with decreased osmotic resistance most likely reflect changes in size and/or shape occasioned by an isolated increase in erythroblastic mitotic activity in the absence of a comparable stimulus to haemoglobin formation.

In view of the evidence which indicates that there are two humoral erythropoietic factors with different natures and physiological activities, the methods used to demonstrate enhanced erythropoiesis in recipient animals and to prepare materials for testing assume paramount importance in the interpretation of experimental results. Thus, the existence of two such factors would appear to explain and reconcile certain discrepancies in reported observations and points of controversy which currently characterize this field of haematological research. The precise chemical identities of these humoral agents are not yet known. However, there is strong support for the contention that the factor which enhances haemoglobin synthesis is a mucoprotein (Rambach, Cooper and Alt, 1958; Gordon, 1959; Lowy *et al.*, 1959). The thermostable, ether-soluble factor which affects cellular division appears to be a lipid (Linman and Bethell, 1960).

The complete solution to the problem of the homeostatic control of erythropoiesis is not yet at hand, but existent data permit certain conclusions. Despite some dissenting opinions, hypoxia, regardless of type, appears to constitute the fundamental erythropoietic stimulus which is mediated to the myeloid reticulum by humoral factors. The relationship between oxygen supply and tissue metabolic requirements apparently determines the level of plasma erythropoietic factor activity (Erslev, 1955; Jacobson et al., 1959). However, the manner by which hypoxia regulates the elaboration or activation of these humoral agents and the site or sites of this effect are unknown.

Haemoglobin synthesis and the maturation of erythrocytic precursors are envisaged as proceeding at fixed and predetermined rates which most likely reflect the inherent growth potential of the rubriblasts to differentiate and mature into non-nucleated, haemoglobin-containing erythrocytes. It is proposed that the humoral erythropoietic factors, through their effects on reticulum cell erythrocytic differentiation and homeoplastic cellular division of erythrocytic precursors, control both the quantity of haemo-

globin produced and the number of erythrocytes in which it is distributed. A well-balanced equilibrium appears to exist for a given level of metabolic activity which ensures, in the presence of sufficient building blocks and an intact myeloid reticulum, not only an oxygen-carrying capacity of the blood commensurate with cellular needs but the production of red cells which are of an optimal size and haemoglobin content for the most efficient completion of their physiological functions. Since concentrates of normal plasma are erythropoietically active (Bethell, Linman and Korst, 1957; Gurney, Goldwasser and Pan, 1957), it may be inferred that the plasma factors contribute to the maintenance of the normal erythroid steady state.

The humoral erythropoietic mechanism is apparently capable of responding even to minor alterations in the dynamic relationship between oxygen supply and tissue requirements. Erythrocytic equilibrium is thereby re-established at the same or a different level, as the situation warrants. The plasma erythropoietic factors are undoubtedly responsible for the increased erythropoiesis which follows haemorrhage or haemolysis. Augmented plasma erythropoietic activity is also demonstrable in patients with anaemias due to impaired erythrocytogenesis secondary to well-established marrow abnormalities or deficiency states. However, the latter preclude a normal marrow response to the humoral stimulus. Although it has not yet been proved, impaired production of the plasma factors may be of pathogenic importance in some currently obscure anaemias.

Experimental observations indicate that these humoral agents also constitute the physiological corrective mechanism which attempts to maintain an adequate supply of oxygen for the tissues in the face of decreased arterial oxygen saturation whether secondary to lowered oxygen tension of inspired air or certain cardiac or pulmonary defects The inability to compensate for severe arterial hypoxaemia eventuates in an unphysiological secondary polycythaemia. This scheme of erythropoietic control

would also include situations which involve changes in the rate of metabolic activity and, therefore, oxygen requirements. Preliminary findings support this concept and indicate that the plasma erythropoietic factors may be of major importance in the pathogenesis of the erythrocytic responses which accompany various states of altered metabolism.

It should be emphasized that the above hypothesis as to the primary regulatory control of erythropoiesis is in part speculative. However, it is fortified by many experimental observations Pending further investigation which will probably necessitate revision of certain current concepts, it provides a logical and rational explanation for the physiological and pathophysiological mechanisms which maintain the normal erythroid steady state and govern the production of red blood cells in a number of abnormal states.

In the light of recent advances in our knowledge of the mechanisms which govern erythropoiesis, studies on the haemopoietic effects of batyl alcohol assume added interest and bear directly on the problem of the regulatory control of haemopoiesis. Batyl alcohol, the monoglycerol ether of *n*-octadecyl alcohol, has been isolated from the non-saponifiable fraction of bovine yellow bone marrow (Holmes *et al.*, 1941) and implicated in erythropoiesis (Sandler, 1949), leucopoiesis (Brohult, 1957), and thrombopoiesis (Evans *et al.*, 1957). The apparent importance of the glyceryl ethers, which include batyl, selachyl, and chimyl alcohols, as biologically active compounds has also been emphasized by discoveries of their wide distribution in nature (Bodman and Maisin, 1958).

Recent studies in our laboratories have confirmed the erythropoietic, thrombopoietic, and granulopoietic stimulatory activity of batyl alcohol (Linman, Bethell and Long, 1958*b*, Linman *et al.*, 1959). Multiple daily doses of racemic batyl alcohol are effective in normal rats by either parenteral or oral routes. This compound induces erythrocytic responses which are identical in all

aspects demonstrable by the methods employed to those otherwise observed only in recipients of the thermostable or ether-soluble fractions of erythropoietically active plasmas, i.e. erythrocytosis due to the production of microcytes with impaired osmotic resistance, reticulocytosis, and myeloid erythrocytic hyperplasia without associated increase in haemoglobin or haematocrit levels. Batyl alcohol also fails to enhance the incorporation of ^{59}Fe in haemoglobin.

In addition to its erythropoietic activity, batyl alcohol exerts other effects on haemopoiesis. Thrombocytosis is evident in normal rats given 12·5 or 25 mg. of batyl alcohol per day for two to four weeks When daily doses of 50 mg. are used, the recipients manifest leucocytosis plus erythromicrocytosis, reticulocytosis, and thrombocytosis. The leucocyte increase is primarily due to a neutrophilia and is accompanied by myeloid granulocytic hyperplasia. All values return promptly to normal base-line levels after the treatment is stopped Under the conditions of these short-term experiments, no adverse effects have been attributed to the administration of batyl alcohol.

For the same reasons applicable in the case of the thermostable, ether-soluble plasma erythropoietic factor, it can be concluded that batyl alcohol also exerts a primary stimulatory effect on erythroblastic cellular division without enhancing haemoglobin synthesis In addition to this unique type of erythropoietic effect, batyl alcohol possesses thrombocytosis- and granulocytosis-promoting properties. However, greater amounts of this substance are apparently needed to induce granulocytosis, at least in the normal rat, than are needed to impart a demonstrable erythropoietic or thrombopoietic stimulus.

A number of points must be clarified before it will be possible to ascertain what rôle, if any, batyl alcohol may play in the control of haemopoiesis Foremost among these is the apparent inconsistency between the dosage of batyl alcohol required to alter myelopoiesis in normal rats, i.e. at least 12 5 mg. daily for

two to four weeks, and the much smaller amounts of an agent possessing such specific biological activity which would be expected to exert a readily detectable effect. Conceivable explanations include, among others, poor absorption with exogenous requirements far exceeding endogenous needs; the use of synthetic racemic preparations rather than optically active, natural material; and the possibility that batyl alcohol may be closely related to but not identical with some other more active substance.

Selachyl alcohol ($C_{21}H_{42}O_3$) differs only slightly in chemical structure from batyl alcohol ($C_{21}H_{44}O_3$) and is the glyceryl ether found most abundantly in nature (Deuel, 1951). Moreover, it is a liquid at room temperature, whereas batyl alcohol is a solid. Consequently, observations on the haemopoietic effects of selachyl alcohol are of interest. Normal rats were given 50 mg of optically active, naturally-occurring selachyl alcohol (kindly supplied by the Squibb Institute for Medical Research, New Brunswick, N.J.) via gastric intubation daily for two weeks. Other rats were given a comparable amount of optically active batyl alcohol (Squibb Institute for Medical Research) by the same route. The latter was prepared by hydrogenation of a sample of the selachyl alcohol given to the former group. The batyl alcohol was suspended in normal saline. A third group of animals received physiological saline solution and served as the controls.

The results of this endeavour are summarized in part in Fig. 5. After treatment for two weeks, the marrow nucleated cell counts in the animals given selachyl alcohol were comparable to those in the control group. However, the recipients of the batyl alcohol exhibited granulocytic and erythrocytic myeloid hyperplasia together with increased numbers of megakaryocytes. These marrow findings were manifested in the peripheral blood by erythromicrocytosis, reticulocytosis, thrombocytosis, and leucocytosis. The haemoglobins and haematocrits remained stable in these animals as did all of the haematological determinations in the recipients of the selachyl alcohol or normal saline. Identical

results have been observed in a similar experimental set-up using a racemic synthetic selachyl alcohol. The responses induced by optically active batyl alcohol did not differ in type or magnitude from those observed with racemic preparations.

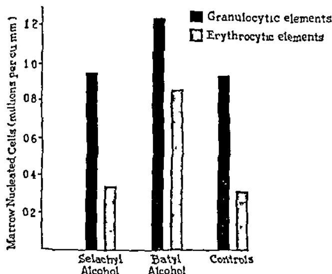


FIG. 5 Average marrow cell counts of eight normal rats given either 50 mg. of selachyl alcohol, a comparable amount of batyl alcohol, or normal saline via gastric intubation daily for two weeks. The marrow findings in the recipients of selachyl alcohol were identical with the controls All haematological determinations remained stable in these two groups. Recipients of the batyl alcohol, prepared by hydrogenation of the selachyl alcohol, manifested erythromicrocytosis, reticulocytosis, thrombocytosis, leucocytosis, and as depicted above, both granulocytic and erythrocytic myeloid hyperplasia

Batyl alcohol is equally effective in normal rats when given orally or parenterally. Moreover, other studies indicate that the glyceryl ethers can be absorbed intact from the gastrointestinal tract (Blomstrand, 1959). However, the above experiments were repeated using a parenteral route of administration in order

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Augmented amounts of both the relatively thermolabile and thermostable plasma erythropoietic factors have been demonstrated in patients with polycythaemia vera. However, the agent which controls erythroblastic cellular division apparently predominates. We have yet to study a thermostable or ether-soluble extract of a "polycythaemic" plasma that has not evoked

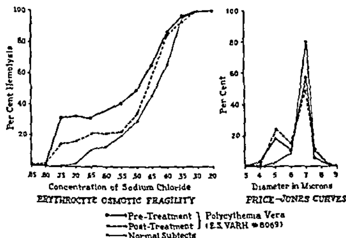


FIG 6. Osmotic fragility curves determined by a direct cell enumeration technique and Price-Jones measurements in a patient with polycythaemia vera. The microcytes with decreased osmotic resistance persisted irrespective of the restoration of normal erythroid values after treatment with ^{32}P. Control values are the composite curves of eight normal subjects (From Linman, Korst and Bethell, 1959. Reproduced from *Ann N.Y. Acad. Sci* by permission.)

a clear-cut erythromicrocytic response in normal rats given multiple injections. However, when tested in the unmodified state or after boiling for less than five minutes, all such plasmas do not induce unequivocally significant increases in ^{59}Fe uptake. Furthermore, microcytes with decreased osmotic resistance are present in the peripheral blood of patients with polycythaemia vera (Fig. 6). These cells are remarkably similar to those observed

to exclude with certainty possible inactivation in the gastrointestinal tract. Subcutaneous injections of 50 mg. of selachyl alcohol daily failed to stimulate any aspect of haemopoiesis in normal rats. Thus, batyl and selachyl alcohol differ markedly in physiological activities in spite of their striking structural similarities.

The true physiological and pathophysiological significance of the haematological phenomena ascribed to batyl alcohol have yet to be elucidated. However, they are in accord with the theory that all aspects of haemopoiesis may be subject to humoral regulatory control and that a single substance or activator-inhibitor complex may govern the proliferation of all haemic elements. In addition, the chemical and physiological properties common to batyl alcohol and the thermostable, ether-soluble plasma erythropoietic factor suggest, reasonably but as yet without proof, that they are closely related. Experimental observations on the rôle of humoral factors in polycythaemia vera provide further support for these concepts.

Enhanced erythropoietic activity is present in the plasmas of patients with polycythaemia vera (Linman and Bethell, 1957*a*; Contopoulos *et al*, 1957) and persists irrespective of the institution of specific myelosuppressive therapy or the level of the peripheral erythroid values (Linman, Korst and Bethell, 1959; Linman, Bethell and Long, 1959). Since the erythropoietic factors do not appear to be elaborated by myeloid elements (Erslev and Lavietes, 1954; Linman and Bethell, 1957*b*), considerable significance must be attached to the increased plasma activity demonstrable in these patients. This finding, which occurs in the absence of hypoxia, the apparent *sine qua non* for enhanced plasma activity in all other situations, is the basis for the thesis that an imbalance or derangement in the humoral erythropoietic regulatory mechanism is of aetiological significance in polycythaemia vera. Other studies suggest that the thrombocytosis and leucocytosis so frequently seen in these patients may be due to a similar mechanism (Linman, Bethell and Long, 1959).

extracts of plasmas from four patients with polycythaemia vera are shown in Figs. 7 and 8. These findings have since been corroborated in groups of rats given comparable amounts of the ether-soluble or thermostable fractions of plasmas from nine additional patients with polycythaemia vera.

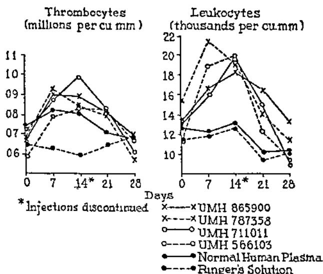


FIG 7. Thrombocytosis and leucocytosis in normal rats given the boiled extracts of plasmas from four patients with active polycythaemia vera Daily injections were equivalent to 4 ml of the original plasma per 100 g body weight Average counts of four animals in each group receiving the above-described materials The leucocyte counts and haematocrits of the plasma donors are shown in Fig 8 Each patient had moderate to marked thrombocytosis (From Linman, Bethell and Long, 1959. Reproduced from *Ann intern Med* by permission)

The magnitude and uniformity of the thrombocyte and leucocyte increases in recipients of these "polycythaemic" plasma extracts together with the marrow findings and rapid return to normal after the injections are stopped would appear to establish beyond reasonable doubt the presence of thrombocytic and

in normal rats (see Figs. 2 and 4) given the thermostable or ether-soluble fractions of plasmas from donors with anaemic, hypoxic, or histiotoxic hypoxia or from patients with polycythaemia vera They are still discernible in polycythaemic patients during therapeutic remissions (Fig. 6). Since the thermostable, ether-soluble plasma erythropoietic factor may be related to batyl alcohol, a substance which exerts a stimulatory effect on the proliferative activity of all myeloid elements, experiments were designed to test the hypothesis that the thrombocytosis and leucocytosis so frequently observed in polycythaemia vera might be the result of excessive humoral factor activity.

To date, we have determined the thrombopoietic and leucopoietic responses in groups of normal rats given ten daily injections of either the thermostable or ether-soluble fractions of plasmas from 17 patients with active polycythaemia vera and seven in therapeutic remission. The amounts of the daily injections, in terms of the original volumes of the plasmas, have varied from two to four per cent of the recipients' body weights. The recipients of each of these plasmas have manifested typical erythrocytic responses, i.e. erythromicrocytosis, reticulocytosis, and myeloid erythrocytic hyperplasia without change in their haemoglobins or haematocrits. In addition, all of these animals have developed thrombocytosis. However, daily doses which were used in our early studies and equivalent to only two per cent of the recipients' body weights did not induce leucocytosis or myeloid granulocytic hyperplasia in normal rats.

Since the studies with batyl alcohol indicated a dose-response relationship, the above experiments were repeated with daily injections of plasma extracts which were one and a half to two times larger. These animals developed leucocytosis, primarily the result of an absolute neutrophilia, and myeloid granulocytic hyperplasia in addition to the erythrocytic and thrombocytic responses previously observed. Examples of the thrombocytosis, leucocytosis, and marrow findings in normal rats given the boiled

parently imparted the maximal stimulus to erythropoiesis and thrombopoiesis. The increases in erythrocyte, thrombocyte, and marrow nucleated red cell counts have been comparable in all recipients irrespective of the size of the daily injection. (3) It has not been possible to quantify the haemopoietic activity of these plasma extracts or to relate either the degree or type of response in the recipients to the magnitude of the erythrocytosis, thrombocytosis, or leucocytosis in the patients. It can be concluded, however, that the myelopoietic effects of these plasmas are not predicated on the presence of active disease in the donors. (4) Although normal plasma extracts were devoid of erythropoietic or granulopoietic activity, these materials appeared to exert a minimal thrombocytosis-promoting effect in normal rats (Fig. 7). The significance, if any, of this finding is conjectural. However, it is compatible with observations on a child with chronic thrombocytopenia (Schulman *et al.*, 1960). Normal plasma has repeatedly but temporarily restored this child's platelet count to normal, thus suggesting the presence of a thrombopoietic factor in normal plasma.

The demonstration of thrombopoietic and granulopoietic effects in the plasma of patients with polycythaemia vera indicates that all of the haematological manifestations of this disease state are the result of augmented humoral factor activity. These findings also provide strong direct support for the hypothesis that all aspects of haemopoiesis are subject to humoral regulatory mechanisms and that a single substance or complex may control the proliferation of all haemic elements. The possibility of multiple factors, each affecting a single cell type, cannot be excluded. However, the thrombocytic and leucocytic stimuli in "polycythaemic" plasmas have not as yet been dissociated from the thermostable, ether-soluble plasma erythropoietic factor which has been studied.

The existence of a *humoral haemopoietic regulatory mechanism* demands more experimental documentation than is currently

granulocytic stimulatory activity in the plasma of patients with polycythaemia vera. Several other points of interest have emerged from these studies: (1) Larger quantities of most "polycythaemic" plasma extracts are needed to induce leucocytosis in recipient animals, whereas smaller amounts have consistently

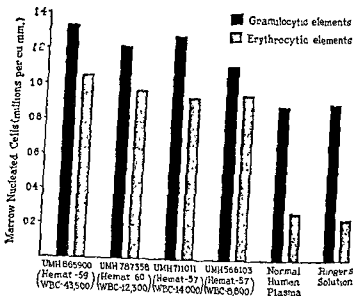


FIG. 8. Increased numbers of both granulocytic and nucleated erythrocytic elements in the marrows of the recipients of the "polycythaemic" plasma extracts which induced the thrombocytosis and leucocytosis depicted in Fig 7 Average counts of two rats given each of the above-described materials. The donors' haematocrits and leucocyte counts are shown in parentheses. (From Linman, Bethell and Long, 1959 Reproduced from *Ann. intern Med.* by permission)

exerted demonstrable erythropoietic and thrombopoietic effects. Thus, there apparently exist, at least in the normal rat, varying degrees of sensitivity or responsiveness of the different myeloid elements to the stimulus contained in these test materials. (2) The smallest daily dosage used in these experiments, i.e. 2 ml. of the original plasma per 100 g. of the recipient's body weight, ap-

gest that these substances may be closely related. Batyl alcohol induces thrombocytosis, leucocytosis, and an increase in marrow granulocytic cell counts in normal rats together with erythromicrocytosis, reticulocytosis, and myeloid erythrocytic hyperplasia. Selachyl alcohol, another glyceryl ether which differs only slightly in chemical structure, is inactive. The thermostable or ether-soluble fractions of plasmas from patients with polycythaemia vera also contain thrombopoietic and granulopoietic stimulatory activity. These phenomena are in apparent accord with the concept that all aspects of haemopoiesis may be subject to humoral regulatory control. It is suggested, although not yet proved, that a single substance may affect the proliferation of all haemic elements and may clarify the pathogenesis of certain poorly understood haematological disorders and responses.

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available. However, it is suggested, on the basis of data now at hand, that a thermostable, ether-soluble agent may affect, although not to the exclusion of other mechanisms, the proliferation of all elements derived from the myeloid reticulum and may explain the thrombocytosis and leucocytosis associated with polycythaemia vera, acute haemorrhage, certain types of haemolytic anaemia, etc. Although the entire subject of haemopoietic control needs further study, the fundamental importance of humoral mechanisms in the regulation of blood cell production can no longer be denied. Continued basic experimentation along these lines should make possible the elucidation of certain currently ill-defined haematological disorders and responses.

Summary

Observations on the erythropoietic activity of certain plasmas indicate that two humoral factors exert regulatory control over erythropoiesis. A thermostable, ether-soluble factor, which is probably a lipid, apparently stimulates cellular division of erythrocytic precursors. A relatively thermolabile, ether-insoluble agent enhances haemoglobin synthesis. The latter appears to be a mucoprotein and most probably induces erythrocytic differentiation of the pluripotential myeloid reticulum cells. It is suggested that these factors control, individually, the number of erythrocytes and quantity of haemoglobin produced and that their combined effects determine, in addition, the size and haemoglobin content of each erythrocyte. Available data support the thesis that this humoral mechanism contributes to the maintenance of the normal erythroid steady state and mediates the fundamental erythropoietic stimulus imparted by hypoxic and certain types of anaemic hypoxia. Increased erythropoietic factor activity appears to be of aetiological significance in polycythaemia vera.

The chemical and physiological properties common to the thermostable, ether-soluble plasma factor and batyl alcohol sug-

you would see some erythropoietin activity in the plasma of such animals at the peak of erythrocytosis.

Linman: Your suggestion of using ^{59}Fe radioautography is an excellent one and such studies have been planned. The possibility that the unique response observed in normal rats given the ether-soluble, thermostable erythropoietic factor might represent an unphysiological or irritative effect must, of course, be excluded. However, the variety of clinical and experimental situations in which this particular erythropoietic effect can be detected, the failure of normal plasma to induce similar changes in the recipient rats, the finding of microcytes with abnormal osmotic behaviour in the peripheral blood of patients with polycythaemia vera, etc., afford strong support for the contention that this factor is physiologically active.

Lajtha: Your experiments demonstrate that it is physiologically occurring. The point is, is this erythrocyte-stimulating factor a second type of erythropoietin, or is it a disturbing factor?

Linman: The marrow findings in the recipients of the thermostable, ether-soluble erythropoietic factor establish the presence of increased proliferative activity of nucleated erythrocytic precursors. Since the microcytes apparently possess impaired viability, the endogenous elaboration of the relatively thermolabile factor undoubtedly plays a rôle in the maintenance of normal haemoglobin levels in long-term experiments. However, the microcytes probably have survival times of approximately 15 days. Furthermore, reticulocytosis can be effected after only three daily injections and at a time when ^{59}Fe uptake is not detectably augmented. It should be emphasized that the production of microcytes in recipients of the thermostable factor alone represents an abnormal experimental situation. We have yet to study an active plasma, regardless of the experimental or clinical conditions under which it was obtained, that has not possessed enhanced activity attributable to both erythropoietic factors. It is suggested that their combined effects are needed for the formation of red cells which are normal in size and haemoglobin content for the species involved.

Cotes: Have you studied plasma from patients with secondary polycythaemia due to pulmonary disease, and does this contain the factor which will increase the platelet count of test rats?

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DISCUSSION

Lajtha: You postulated that the ether-soluble factor might induce an extra mitosis, or stimulate mitosis, and therefore produce cells with half the haemoglobin content. This would naturally result in no change in the iron appearance curve, no change in haemoglobin, but an increased red cell count If this is so, I would suggest that you can test it quite clearly by giving ^{59}Fe, and afterwards giving a single injection of your ether-soluble factor. You should get a decrease in the grain count of the appearing reticulocytes as compared to the control. If that is not the case, I would tentatively suggest that this compound may be nothing but a non-specific toxic agent for the cells, which therefore cannot work properly and some of them just turn into microcytes. This, of course, may produce eventually a mild anaemia with all its consequences: erythropoietin production, reticulocytosis. This could also be checked with a ^{59}Fe uptake study. In the latter case maybe

in all erythroid values owing to the production of increased numbers of normal-sized erythrocytes. Microcytes are not demonstrable in animals given both humoral factors. It is not suggested that the thermostable factor causes an extra cellular division but rather that it is needed to maintain a uniform number of mitotic divisions during maturation of erythrocytic precursors regardless of the total output by the marrow. In other words, it is proposed that both factors are needed for the formation of normal cells.

Stohlman: You don't get the extra division if you give them both together?

Linman: When both humoral factors are given to a recipient animal, the relatively thermolabile factor apparently augments the number of rubriblasts derived from the pluripotential reticulum cells. Since erythrocytic precursors are increased in this manner, it is postulated that greater amounts of the thermostable factor are then needed in order to maintain a constant number of cellular divisions. It is proposed that the well-balanced effects of both factors determine not only the quantity of haemoglobin and number of red cells produced but also the size and haemoglobin content of each individual cell. An extra mitotic division over those which would occur normally during maturation is hypothesized only in those experimental situations involving increased activity of the thermostable factor alone. The opposite of this experimental set-up would be to give the relatively thermolabile factor alone Theoretically, macrocytes might then be expected. This phase of the problem needs further study but certain preliminary observations indicate that such may be the case.

Braunsteiner: You said that when you inject your erythropoietin you get reticulocytosis, microcytosis, and decreased resistance. We generally get macrocytosis with reticulocytosis and an increased resistance. Your findings are unusual from the clinical standpoint.

Linman: It should be re-emphasized that the erythropoietic response induced in normal rats by the thermostable, ether-soluble factor represents an experimental situation which does not appear to have a clinical counterpart. Since microcytes with decreased resistance to lysis in hypotonic media are not demonstrable in the presence of increased amounts of both erythropoietic factors, these cells cannot be assumed to reflect a specific property of the thermostable factor. The abnormal

Linman: As yet, we have not had a chance to evaluate the effects of plasmas from patients with arterial hypoxaemia and secondary polycythaemia on the platelet or white counts of recipient rats. Data in this regard are certainly needed, and such experiments are planned for the near future.

Cronkite: Is the batyl alcohol synthetic or biological in origin? Which rat are you using? How are the platelets counted? How is the batyl alcohol given that produces the thrombocytes?

Linman: We have studied both optically active batyl alcohol from natural sources and racemic, synthetic preparations. No differences have been noted in either the type or degree of response induced in recipient rats by these two forms of batyl alcohol. Wistar strain rats have been most extensively used in our experiments. However, a number of other strains have been used on occasion and have manifested identical responses. Platelets are counted by a haemacytometer with a phase microscope. A one per cent solution of ammonium oxalate is used as the diluent Batyl alcohol is active both parenterally and orally, but administration by gastric tube is preferred. The rats tolerate daily gastric intubations without untoward effects, and this route of administration obviates changes which might be secondary to tissue reactions at the sites of subcutaneous injections.

Osmond. We had a similar experience to yours with batyl and selachyl alcohol in the smaller doses. We have used doses of 10 mg/kg. daily for five days, subcutaneously, made up in arachis oil. The source of batyl alcohol in this case was from dog-fish liver oils. In this dosage we too noticed an increase in the peripheral reticulocytes, with an increase in the erythroid and lymphocyte population in the marrow, but at that level no increase in the granulocyte precursors We also found the selachyl alcohol quite inactive in all respects.

Stohlman: If I understand correctly, Dr Linman, you suggest that the heat-labile factor stimulates differentiation of a stem cell, and the heat-stable factor stimulates an extra division If you give fresh material that has not been heated, do you develop a normocytic or microcytic polycythaemia? I should think you would have to develop a microcytic polycythaemia because you would still be giving the heat-stable factor.

Linman: Recipients of unmodified active plasmas develop an increase

SOURCES AND PROPERTIES OF HUMAN URINARY ERYTHROPOIETIN*

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THE widespread interest in the physiology, clinical significance, and chemical nature of the erythrocyte-stimulating factor (erythropoietin) found in the plasma and urine of severely anaemic animals and human beings has created a demand for large quantities of active material (symposium on Hematopoietic Mechanisms, 1959). At present, erythropoietin can only be obtained in reasonably large amounts either from the plasma and urine of animals made severely anaemic by bleeding or treatment with phenylhydrazine (White and Josh, 1959) or from the urine of a small proportion of patients with severe anaemia.

An occasional anaemic patient will consistently excrete such a high titre of erythropoietin in the urine that administration of as little as 1 ml. per day to normal rats for 14 days will make them polycythaemic (Van Dyke, Garcia and Lawrence, 1957). Such a patient provides a convenient source of erythropoietin for study since the activity is retained for long periods when the urine is frozen and stable concentrates with fairly high specific activity can be obtained by the relatively simple procedures of adsorption on collodion gel ("ultra-filtration", Van Dyke, Garcia and Lawrence, 1957) or kaolin (Gordon *et al*, 1959). This report deals with the sources, one method of preparation, and some of the properties of human urinary erythropoietin.

* This work was supported in part by the U S Atomic Energy Commission.

osmotic behaviour is most probably due to alterations in cell size and/or shape occasioned by an isolated stimulus to mitotic division of differentiated erythrocytic precursors in the absence of a comparable stimulus to the formation of rubriblasts from the multipotential reticulum cells.

Craddock: Your observation of elevation of white count and platelets after ESF administration was with polycythaemic plasma. Have you compared this with anaemic plasma?

Linman: These experiments are now in progress. Preliminary observations indicate that phenylhydrazine-induced anaemic rabbit plasma is capable of evoking thrombocytosis in normal rats, but definitive conclusions must await further study. Data in regard to the possible granulocytosis-promoting property of "anaemic" plasma are not yet available.

Types of cases excreting erythropoietin

In an attempt to determine the clinical and pathological significance of erythropoietin, the plasma and urine of all available patients with haematological disease have been assayed by the ^{59}Fe red cell incorporation method. Erythropoietic activity has been found only in the presence of severe anaemia and only in a small percentage of the severely anaemic group. There are three categories of activity found in the urine of severely anaemic

Table I

RESULTS OF ERYTHROPOIETIN ASSAY OF PLASMA OR URINE IN VARIOUS HAEMATOLOGICAL DISORDERS

	Number of cases	
Diagnosis	*Positive*	*Negative*
Hypoplastic anaemia	14	9
Leukaemia	3	10
Haemolytic anaemia	2	10
Polycythaemia vera	0	12
Polycythaemia, secondary	0	10
Iron deficiency anaemia	1	6
Kidney disease	0	6
Hypoxia, altitude	0	5
Neoplastic disease	0	4
Maturation defect	2	2
Myelofibrosis	1	1
Anaemia of undetermined origin	0	4
Myxoedema with anaemia	0	1
Pernicious anaemia	0	1

patients: those that have no measurable activity even when large amounts of urine are extracted, those that have small but definite activity, and those few individuals whose urine contains a very high titre of erythropoietin. The latter excrete approximately ten times as much erythropoietin as the intermediate group. Table I indicates the types of cases which have shown measurable erythropoietic activity in the urine. Out of 104 patients (75 anaemias) with haematological disorders who were assayed, only

Method of assay and comparison of dosage

The erythropoietic activity of the samples was determined either by the ^{59}Fe red cell incorporation assay (Plzak *et al.*, 1955) in starved or normal rats, as indicated in the tables, or by the increase in total circulating red cell volume after 14 injections into normal adult female rats fed a complete diet.* The details of the assay as used in this laboratory have been given in a previous publication (Garcia and Van Dyke, 1959).

The specific activity of the various preparations was estimated from the previously published dose-response curves obtained by injecting graded doses of material collected by ultrafiltration of urine from the patient used in the majority of these studies (Garcia and Van Dyke, 1959).

Some basis for comparison of the activity obtained from various sources and in different laboratories is recognized as being essential. Goldwasser and White (1959) have proposed a unit based on the response obtained with a standard dose of cobalt, and Keighley's group at the California Institute of Technology have distributed samples of a single batch of partially purified materials from phenylhydrazine-treated rabbit plasma to a number of different laboratories to provide a comparison (to be published). This comparison indicated that 1 mg. of the human urinary erythropoietin obtained by ultrafiltration and used for the published dose-response curves (Garcia and Van Dyke, 1959) was equivalent to seven cobalt units. In this report, where the dose has been estimated in cobalt units, it has been obtained by comparing the activity of the material used to the published dose-response curve for human urinary erythropoietin and converting to cobalt units by multiplying the dose in mg. equivalents by seven.

* The diet consists of 67·5% whole wheat, 15% casein, 10% whole milk powder, 0·75% NaCl, 1·5% $CaCO_3$, 5·25% hydrogenated vegetable oil, and a concentrate of fish oil in amount to give 19 U.S.P. units of vitamin A and 2·5 A.O.A.C. (Association of Official Agricultural Chemists) chick units of vitamin D per gram of diet.

Table II

ERYTHROPOIETIC ACTIVITY OF URINE FROM PATIENT WITH PAROXYSMAL ERYTHROID APLASIA

Condition	*Assay*	*Dose*	*Result*
Complete aplasia	^{59}Fe uptake in normal female rats	1 ml. native urine per day for 2 days	36·1% uptake*
During remission	^{59}Fe uptake in normal female rats	Equivalent of 21 ml urine/day for 2 days, concentrated by ultra-filtration	42·6% uptake*
During remission	Red cell volume increase in normal female rats	2 86 mg /day for 14 days, urine concentrated by ultrafiltration	Body wt / T.R.C.V.† / % Increase Injected 219 / 8·03±0·5 / 65·5 Control 214 / 4·85±0·1
After recovery	^{59}Fe uptake in normal female rats	1 ml native urine per day for 2 days	21 2% uptake*

* ^{59}Fe uptake for 100 normal controls was 26·1%.
† Total circulating red cell volume ± standard error of the mean.

four individuals showed a very high titre. One was an infant with haemolytic anaemia and splenomegaly and one was a case of paroxysmal erythroid aplasia with hypogammaglobulinaemia.

The case of haemolytic anaemia with splenomegaly was a 6½-year-old Caucasian female who had fused ribs and a congenital tracheo-oesophageal ... der ... ool ... g. ... ia.

Bone marrow aspiration revealed an increase in erythroid activity with a definite relative increase of immature forms. Erythropoiesis was of the normoblastic type. Granulocytopoiesis was decidedly reduced and only one megakaryocyte was seen on several preparations. Reticuloendothelial cells, plasma cells and tissue mast cells were quite numerous in this sample. The total circulating red cell volume was 8·9 ml /kg. body weight (normal 24-33) Analysis of plasma radioiron * showed a daily formation of 4·5 g. of haemoglobin When related to the total body haemoglobin, which was 89 g., this indicated a mean lifespan of 20 days. *In vivo* measurements showed accumulation and normal subsequent release of radioiron from the sacral marrow followed by a secondary accumulation of radioiron in the spleen and liver, indicating some splenic and hepatic sequestration and destruction of erythrocytes Marrow erythropoiesis was increased approximately two and a half times, which was ... in mean erythrocyte lifespan.

... Hall, San Francisco.)

The case of paroxysmal erythroid aplasia with hypogammaglobulinaemia was a Caucasian male of 19 years at the time of study. Three paroxysms of ... years of ...

... second episode of aplasia were as follows: haemoglobin, 4 g., haematocrit, ... volume, ... owed the ... 2·955 g. after remission. Assay for urinary erythropoietin was done before, during, and following remission in the second episode of aplasia (Table II). As can be seen from the table, a high level of urinary erythropoietin was found before and

* Iron kinetic studies were done by Dr. Myron Pollycove of this laboratory.

the plasma and urine of four patients with haemoglobin of 3·5 to 6·5 g. secondary to hookworm infection (*Ancylostoma necator Americanus*) and found no activity in either the native urine or urine concentrated by adsorption on collodion gel.

One patient who had polycythaemia associated with a clear cell carcinoma of the kidney showed no measurable erythropoietic activity in the plasma or urine or saline extracts of the tumour or kidney tissue. An alcohol fractionation of 200 g. of tissue, containing approximately equal amounts of tumour and

Table III

ERYTHROPOIETIC ACTIVITY OF FRACTIONS OF "POLYCYTHAEMIA-PRODUCING" KIDNEY TUMOUR
(10 rats in each group)

Alcohol concentration	^{59}Fe *uptake* (%)
26–35	6·1
35–49	10·2 ± 1·6*
49–60	8·2
60–70	8·9
70–90†	4·9
Uninjected control	5·2 ± 1·4

* Standard error of mean.
† 5 vol acetone added

what remained of the kidney, was made in an attempt to concentrate any small amount of erythropoietin that might be present. The results of the assay of these fractions are presented in Table III. The fraction which precipitated between 35 and 49 per cent alcohol concentration produced a small but definite increase in ^{59}Fe incorporation.

In an attempt to clarify the rôle of the kidney in erythropoiesis, the response to hypoxia of nephrectomized animals maintained by parabiosis to normal partners has been studied.

Since one of the members can be maintained by the kidneys of its partner, a nephrectomized animal can be kept in good health indefinitely and one can test its ability to respond to hypoxia

during remission, but none was found after recovery when the haemoglobin had returned to normal. During the third episode of aplasia, serial electrophoretic studies demonstrated a rise in γ-globulin as the erythrocyte values returned to normal.*

A third case with erythroid hypoplasia, summarized previously (Van Dyke, Garcia and Lawrence, 1957), was an excellent source for two years and then suddenly dropped into the intermediate category; the activity of her urine fell to one-tenth of what it had been for the previous two years without apparent change in clinical condition. Examination of the urine of this patient by Dr. K. I. Altman† has shown an abnormally high level of 3-hydroxykynurenine indicating a derangement of tryptophan metabolism of the type associated with erythroid hypoplasia in erythrogenesis imperfecta (Dalgliesh, 1955). A fourth patient (with paroxysmal nocturnal haemoglobinuria), summarized previously (Van Dyke, Garcia and Lawrence, 1958), remained active for the two years during which his urine was collected, but was lost to us when he returned to his home after graduation. Both of the patients who were consistently good sources maintained a haemoglobin concentration of approximately 5 g. One had an almost complete absence of red cell production whereas the other had a high rate of red cell production. The urine of these two cases was the source of erythropoietin for these studies.

Because of the marked dependence of erythropoietin titre on haematocrit or haemoglobin concentration demonstrated in rabbits (Lowy *et al.*, 1959), sheep (White and Josh, 1959), dogs (Naets, 1959), and man (Gordon *et al*, 1959), we have considered where a large population of patients with haemoglobin of 5 g. or below might be found. A large group of severely anaemic patients is found in tropical areas where hookworm flourishes. Through the co-operation of Dr. M. Layrisse,‡ we have assayed

* Case to be reported in detail by Dr Joseph Linsk, Atlantic City, New Jersey.
† University of Rochester, New York
‡ Cases to be published in detail by Dr Miguel Layrisse, Banco de Sangre del Distrito Federal, Caracas, Venezuela

Table IV

RESPONSE TO HYPOXIA OF NEPHRECTOMIZED RATS AS DEMONSTRATED WITH PARABIOSIS

Combination	O_2 *in atmosphere* (%)	*Blood urea nitrogen* (mg %)	% ^{59}Fe *uptake in* *Spleen*	*Liver*	*Bone marrow*	*Red cells*	*Plasma iron* (µg /100 ml)	^{59}*Fe uptake* × *plasma iron concentration*
Nephrectomized	21	100	0·31	2·1	5 0	16·7	47	8 01
Normal*	21	18	1·06	4·1	12 9		49	
Nephrectomized*	21	185	0·63	8·5	13 6	8·8	105	9·32
Normal	21	24	0·54	3·4	6·1		108	
Nephrectomized	21	33	1·30	0·5	3 0	61 0	78	40·3
Normal*	10	7	1·50	4·3	16·7		53	
Nephrectomized*	21	89	2·2	3·2	17·5	73·2	51	63·9
Normal	10	22	1 1	0·9	3·5		124	
Nephrectomized*	10	52	0 60	9·8	23·1	20 9	187	44·3
Normal	21	18	1·40	3·7	21·0		236	
Nephrectomized	10	46	0 5	6 5	10·0	16 6	114	18 7
Normal*	21	21	0·5	10·6	17 2		111	
Nephrectomized*	10	47	0 6	5 5	15·4	35·4	228	76·5
Normal	21	21	0·8	2·7	10·4		204	

* Injected with ^{59}Fe

using the classic technique of Reissman (1950). Inbred rats of the Fisher strain were used. One member of several pairs of adult rats which had been parabiosed for one month was nephrectomized. Three days after nephrectomy either the normal or the nephrectomized partner was subjected to an atmosphere of 10 per cent oxygen for 12 hours on three consecutive days. On the following day ^{59}Fe was given intravenously and 18 hours later blood was drawn for counting and urea nitrogen determination. The rats were perfused with saline, and liver, spleen, and marrow samples were taken for counting. The results are summarized in Table IV. The incorporation of ^{59}Fe into spleen, liver and marrow was usually greater in the animal to whom the iron had been given whether it was normal or nephrectomized, indicating that this degree of elevation of the blood urea nitrogen *per se* does not inhibit erythropoiesis. This has been demonstrated in ureter-ligated animals by Goldwasser, Fried and Jacobson (1958). The ^{59}Fe red cell incorporation corrected for the plasma iron concentration indicates that the response of nephrectomized rats to hypoxia was equal to that of the non-nephrectomized controls. As can be seen from the table, this result depends entirely on the validity of the plasma iron concentration which was measured at the end of the experiment, 18 hours after administration of the ^{59}Fe.

Nephrectomized animals maintained in good health by parabiosis with normal partners appear to be an ideal approach to the question of the renal origin of erythropoietin. One difficulty with the use of ^{59}Fe as an indicator of erythropoiesis in such a combination is the fact that plasma for iron determination cannot be taken at the time of administration of ^{59}Fe in such small animals without disturbing the equilibrium. However, the results of the preliminary studies using such animals, and assuming that the plasma iron level does not markedly change in the 18 hours between administration of the ^{59}Fe and autopsy, indicate that nephrectomized rats in parabiosis with normal partners show a normal erythropoietic

material have demonstrated that, as is the case with urinary gonadotrophin (Van Gilse, 1955), recovery of the activity results both from the process of retention of material because of pore size and from adsorption to the collodion gel of the membrane through which the urine is filtered.

It was observed that when one of the collodion membranes was immersed in erythropoietically active urine overnight and processed by dissolving in ether-alcohol, erythropoietic activity was

Table V

COMPARISON OF ERYTHROPOIETIC ACTIVITY RECOVERED WITH DIFFERENT AMOUNTS OF ADSORBENT WITH THAT RECOVERED BY ULTRAFILTRATION (10 starved rats in each group)

No. of membranes	*Amount recovered* (mg)	*Daily dose* (mg)	^{59}Fe *uptake* (%)	*Specific activity**
Ultrafiltration	24·0	1·0	31·4 ± 1·9†	0·9
2	29 0	1·21	22·7 ± 2·1	0·2
1	19·0	0 79	15 0 ± 0·4	0·2
1/2	6·0	0 25	11·3 ± 3·9	0·3
1/4	3 5	0·15	7·5 ± 0·8	0·4
Uninjected control			3·9 ± 1·3	

* Specific activity as compared to the previously published dose-response curve (Garcia and Van Dyke, 1959)
† Standard error of the mean

recovered from the membrane. When various amounts of collodion (from a quarter to two membranes) were immersed in 300 ml. samples of urine for two hours, the weight of material adsorbed was proportional to the amount of membrane added and the erythropoietic specific activity was considerably lower than that obtained by ultrafiltration. The results are summarized in Table V.

The effect of time of exposure of the membrane on specific activity and amount of recovered material was tested in the following way. A half membrane was added to each of four 650

response to hypoxia. This cannot be called a response in the absence of the kidneys but a response in the absence of hypoxic kidneys since it is conceivable that the kidneys of the normoxic partner could be stimulated to produce erythropoietin through some mechanism other than hypoxia.

Method of collection and preparation

Those patients whose urine was consistently very high in activity were provided with freezing units in their quarters so that a large part of their output could be collected. The urine was frozen immediately after voiding and the frozen urine was delivered once a week. One adult patient can conveniently collect approximately 500 ml. per day or 15 litres a month, from which one obtains one gram of active powder by ultrafiltration. Approximately 1,500 ml. of urine were thawed and filtered through coarse filter paper before ultrafiltration. The collodion membrane was prepared as described by Gorbman (1945). Each membrane was made with 10 ml. of a 2 per cent nitrocellulose solution prepared as follows: seven parts absolute ethanol, three parts absolute ethyl ether, one part glacial acetic acid and 11 parts U.S.P. collodion (Merck). After the urine had been forced through the membrane under 20 lb. pressure from a nitrogen tank, the membrane was placed in a centrifuge tube, dissolved in ether-alcohol, and centrifuged. The supernatant containing the dissolved membrane was discarded, and the residue was washed twice with ether-alcohol (50–50), once with ether, dried and stored under vacuum. Approximately 50 per cent of the activity remains in the insoluble portion when such material is dissolved in cold water, but the activity can be brought into solution by immersing for half to one minute in boiling water. It was this material that was used for all studies except where otherwise indicated.

Although the technique of ultrafiltration has proved to be a satisfactory first step for recovering erythropoietin from human urine, attempts to improve the specific activity of the recovered

Table VII

COMPARISON OF THE ERYTHROPOIETIC ACTIVITY OF THE ADSORBED MATERIAL WITH THAT WHICH COULD BE OBTAINED BY REVERSING AND WASHING THE MEMBRANE
(6 normal rats in each group)

Source	*Material*	*Dose* (mg/day/14 days)	*Haematocrit* (%)	*Haemoglobin* (g)	*Total red cell volume* (ml)	% *Increase*
Aplastic anaemia	Filtered	0·9	42·9	12·9	4·99±0·16*	8·0
	Adsorbed	0·9	55·5	15·3	7·44±0·58	61·4
Paroxysmal nocturnal haemoglobinuria	Filtered	2·1	48·1	13·6	5·48±0·11	18·8
	Adsorbed	2·1	58·8	15·3	8·05±0·10	74·6
Uninjected control			46·2	13·3	4·61±0·31	

* Standard error of the mean.

ml. samples of urine with stirring in the refrigerator for 1, 6, 11, and 24 hours; the membranes were then lifted out and dissolved in ether-alcohol. It was found that the greatest weight of material recovered occurred at 11 and 24 hours, but that within the limits of the assay, all fractions had similar specific activity.

From these results it appears that to recover erythropoietin quantitatively by suspending the collodion membrane in the urine would require long exposure of a large surface area and the specific activity of the recovered material would be less than that

Table VI

SEPARATION OF ADSORBED AND FILTERED COMPONENTS OF "ULTRAFILTERED" SAMPLE BY REVERSING AND WASHING MEMBRANE
(10 starved rats in each group)

Material	*Amount recovered* (mg)	*Daily dose* (mg)	^{59}Fe *uptake* (%)	*Specific activity**
"Ultrafiltration"	37	1·54	43 2±2·2†	1·3
Adsorbed	30	1 25	42·7±5 1	1·6
Filtered	8	0 33	7·2±1·6	0
Uninjected control			5 6±0·9	

* *Specific activity as compared to the previously published dose-response curve* (Garcia and Van Dyke, 1959).
† *Standard error of the mean*

obtained by ultrafiltration, suggesting that ultrafiltration provides an ideal method of exposing the urine to the adsorbent. However, the material recovered by ultrafiltration represents both adsorbed material and that which is retained because of the pore size. A simple method of separating the two materials is ultrafiltration followed by reversing the membrane and forcing through deionized water to wash away the non-adsorbed material. Table VI compares the results obtained by ultrafiltration with ultrafiltration followed by reversing the membrane and washing with deionized water. The washing was concentrated by a second ultrafiltration. As can be seen from the table, the total

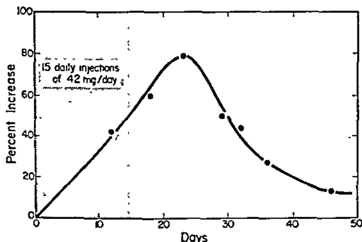


FIG. 1. The most marked increase in total circulating red cell volume obtained in monkeys with human urinary erythropoietin. Note that the red cell volume continued to increase for one week after the last injection

Table VIII

POLYCYTHAEMIA IN NORMAL MONKEYS AFTER 15 DAILY INJECTIONS OF HUMAN URINARY ERYTHROPOIETIN

Daily dose		*Body weight*	*% Increase in*		
mg. equiv.*	cobalt units	(kg)	Haemoglobin	Haematocrit	T.R.C.V.†
90	630	6·6	33	26	61
42	294	2·0	35	41	78
21	147	2·0	3	4	55

* As compared to published dose-response curve (Garcia and Van Dyke, 1959).
† Total circulating red cell volume.

Discussion

The types of cases in which measurable amounts of erythropoietin have been found have had in common only the fact that they were severely anaemic. Many more cases with equally or more severe anaemia have shown no erythropoietic activity. The

amount recovered was the same (37 mg. for ultrafiltration and 38 mg. for the filtered plus adsorbed fractions) and the activity was entirely in the adsorbed portion which represented the majority of the recovered material. The specific activity of the material recovered by ultrafiltration and washing was consistently higher than that recovered by suspending the adsorbent in the urine or by ultrafiltration without subsequent washing.

The technique of reversing and washing the membrane after ultrafiltration was applied routinely to the urine collected over a three-week period in order to get an average value for the proportion of filtered and adsorbed material. The filtered material which was washed from the membrane was recovered by a second ultrafiltration. When eight separate processings were combined, the dry weight of the adsorbed material was two-thirds of the total. When the two fractions from different patients were assayed either by the ^{59}Fe red cell incorporation assay or by the increase in total circulating red cell volume after 14 injections, the erythropoietic activity was found almost entirely in the adsorbed fraction (Table VII). The specific activity of the adsorbed fraction was, in both cases, too high to be compared to the standard curve previously published.

Effectiveness in primates

In an attempt to determine how much erythropoietin would be required for therapeutic trial in human beings, three dose levels of collodion-adsorbed human urinary erythropoietin have been given intravenously, one injection daily, to cynamolgous monkeys for 15 days. The total circulating red cell volume before, during and after treatment was determined by the ^{32}P-labelled red cell dilution method (Berlin *et al.*, 1949). Fig. 1 illustrates the most marked response obtained. In each case the red cell volume continued to increase for several days beyond the time of the last injection. The haematological values at the peak of the response are presented in Table VIII.

hydrazine-treated rabbits. Although no direct comparisons have been made, the method of kaolin adsorption as reported by Gordon and co-workers (1959) appears to be another simple and efficient method for the initial extraction of erythropoietin from human urine.

If one is willing to ignore the differences in route of administration and timing and compare the responses obtained in monkeys

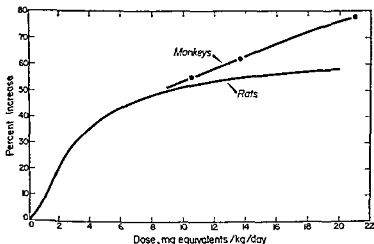


FIG 2. A comparison of the percentage increase in total circulating red cell volume of monkeys and rats in terms of dose of human urinary erythropoietin per kg. body weight per day The dose-response curve for rats was re-drawn from the previously published curve (Garcia and Van Dyke, 1959).

with those obtained in rats, the response in terms of dose per kg. body weight is similar (Fig. 2). The dose required to produce a 50 per cent increase in total circulating red cells in either rats or monkeys is approximately 8·75 mg /kg. of body weight per day for 14 days. On this same basis, a 70 kg. man would require 600 mg./day (or 4,200 cobalt units/day) to produce a 50 per cent increase in red cell volume, or roughly 0 5 g. per day of human urinary erythropoietin. Since it takes one month to collect one

few cases which have shown very high levels of erythropoietin have been entirely different diseases and have had no common aetiological basis which has been recognized. However, the fact that three of the four had evidence of other inborn errors as indicated by congenital anatomical abnormalities in one, hypogammaglobulinaemia in another, and a derangement of tryptophan metabolism as indicated by an abnormally high level of excretion of 3-hydroxykynurenine in the third, suggests that extremely high titres may result from a combination of metabolic defects. An abnormally high production of erythropoietin in response to the anaemia, combined with an abnormality in the removal of the hormone from the circulation, could be responsible for the very high levels occasionally seen. Such a combination has been suggested to account for the higher erythropoietin levels seen following phenylhydrazine treatment than following haemorrhage (Jacobsen, Davis and Alpen, 1956).

The relatively simple procedure of passing the entire urine output from two anaemic patients through a collodion membrane (ultrafiltration) has provided gram quantities of erythropoietic concentrate with a minimum effective daily dose in rats of less than 0·1 mg. in the ^{59}Fe red cell incorporation assay and 0·25 mg. in the 14-day total circulating red cell volume assay (Garcia and Van Dyke, 1959). By reversing the membrane and washing with water following ultrafiltration, 20 to 40 per cent of the solids retained by the membrane can be washed off and discarded as inactive. This relatively small gain in specific activity is of importance when one is dealing with material which was already of sufficient activity and of sufficiently low toxicity to be given in effective amounts to a large variety of species without evidence of toxicity. A comparison of the activity recovered from these patients with that obtained from plasma of phenylhydrazine-treated rabbits (comparisons done in collaboration with Dr. G. Keighley) showed that one very active patient provides as much erythropoietin a day as can be obtained from two or three phenyl-

monkeys and man shows that they are remarkably similar, indicating that extrapolation of dosage from monkey to man may be justified. Fig. 3 compares the changes in total circulating red cell volume during and following exposure to a simulated altitude of 15,000 feet of one of the monkeys (the larger) used in the erythropoietin assay to the changes found in human beings moved from Morococha (14,900 feet) to Lima, Peru (Reynafarje, Lozano and Valdivieso, 1959).* The similar rate of fall of the red cell volume of both man and monkey after prolonged exposure to hypoxia suggests that the mean lifespan of monkey erythrocytes is similar to that of man. An incidental observation was that both the human and monkey data suggest the possibility that a period of relative anaemia follows the return of the red cell volume to normal.

It is possible, as in the case of growth hormone (Knobil *et al.*, 1957), that material of primate origin must be used in order to obtain a response in primates. Until it has been demonstrated that erythropoietin from rabbits or sheep is effective in primates, it seems worth while to search for the rare human being who excretes sufficient erythropoietin in his urine to provide sufficient material for further investigation. At present there is no apparent method for deciding which patient might excrete large amounts of erythropoietin other than to assay the urine of all severely anaemic patients.

Summary

Erythropoietic activity has been found only in the presence of severe anaemia and only in a small percentage of severely anaemic patients.

An occasional anaemic patient will excrete such high titres of erythropoietin in the urine that administration of as little as 1 ml. per day to normal rats for 14 days will make them polycythaemic.

* The percentage change in human beings was based on data given by Reynafarje compared to the average sea level values from the Donner Laboratory clinic (24–33 ml./kg. body weight)

gram from one very active patient, then it would take the total collection from one such patient over a seven-month period to obtain enough human urinary erythropoietin to expect to produce a 50 per cent increase in total circulating red cells in a normal human recipient in 14 days. Since a 25 per cent increase

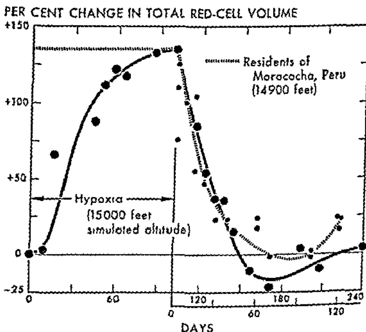


FIG. 3 The changes in total circulating red cell volume during and following exposure to a simulated altitude of 15,000 feet of one of the monkeys (the larger) used in the erythropoietin assay as compared to the changes found by Reynafarje, Lozano and Valdivieso (1959) in humans beings moved from Morococha to Lima, Peru

requires only one-third that dose in rats, one might be able to produce a significant increase in a normal human subject with 200 mg./day (1,400 cobalt units) for 14 days, or the total output of a single active patient for two or three months.

A comparison of the magnitude of response to hypoxia of

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DISCUSSION

Lajtha: In those animals which are difficult to handle in terms of the iron disappearance curve, couldn't one use the dilution of labelled erythroblasts with unlabelled cells as an index for increased erythropoiesis? The rate of feed of unlabelled cells into the labelled compartment is in a sense the rate of differentiation of stem cells into the erythron. E. L. Alpen and D. Cranmore (1959. *In* The Kinetics of Cellular Proliferation, p. 290) have shown very nicely that this gets very steep after bleeding. So if in any animal, for technical reasons—weight loss and so on—you cannot do a reliable iron disappearance curve, take serial bone marrow samples after an initial ^{59}Fe injection. Charting of the proportion of labelled pronormoblasts or basophilic normoblasts will give the rate of feed and therefore the rate of differentiation of stem cells. This would also eliminate complicating influences such as destruction of nucleated red cells while they are in different stages of maturation. You will get a measure of stem differentiation.

Van Dyke Does that get you away from changes in the general iron pool?

Lajtha: Yes. It does not matter where iron goes, the important thing is that when you give the iron initially you should give enough to get a radioautograph. The rest does not matter.

Craddock: Don't you have to give a great deal?

Lajtha: That depends on the specific activity of your ^{59}Fe. You have to give something like 15 µc/200 g. rat.

Lamerton: It is a much more difficult job, of course, than just doing a blood uptake of ^{59}Fe.

Stohlman: It seems to me that these differences are so great that it would be hard to explain them on a basis of changes in the serum iron—these are almost tenfold differences.

The erythropoietic activity of such urine can be concentrated in stable form by ultrafiltration and adsorption on collodion gel. From one very active patient as much erythropoietin can be collected per day as can be obtained from the plasma of two or three phenylhydrazine-treated rabbits; this represents approximately 7,000 cobalt units per month.

Human urinary erythropoietin has been shown to be effective in a variety of species including monkeys. The dose required to produce a 50 per cent increase in total circulating red cells in either rats or monkeys is approximately 60 cobalt units per kg. of body weight per day. On this same basis, a 70 kg. man would require 4,200 cobalt units per day for 14 days to produce a 50 per cent increase in total circulating red cell volume.

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in Bethesda have reported erythropoietic-stimulating activity in fluid aspirated from a cystic cerebellar haemangioblastoma. Might it not be possible that a variety of tissues possess a latent ability to produce erythropoietin and that alteration in their vascular pattern and degree of oxygenation or an abnormal change in their metabolic state might make this ability manifest? Thus polycythaemias are known to occur in some patients with ovarian tumours and uterine fibroids as well as in subjects with certain renal involvements and cerebellar haemangioblastomas.

Yoffey: We need a pathologist to tell us something about the circulation through haemangioblastomas in general and cerebellar haemangioblastomas in particular. Is it the normal cavernous type of circulation or any special form?

Linman: Korst and his associates have reported in abstract form (Korst, D. R., Whalley, B. E., and Bethell, F. H [1959]. *J. Lab. clin. Med*, 54, 916) the study of a patient with hypernephroma and polycythaemia. They were able to demonstrate the presence of a substance in the saline extract of this tumour, as well as in the patient's plasma, that enhanced ^{59}Fe incorporation in recipient rats Following removal of the hypernephroma, the enhanced plasma erythropoietic activity disappeared and the patient's erythroid values returned to normal. Extracts of a hypernephroma from a second patient without associated erythrocytosis were inactive in so far as their ability to enhance ^{59}Fe uptake was concerned.

Stohlman: In my experience if cerebellar haemangioma and hypernephroma are removed the polycythaemia disappears. In most instances where we have seen metastatic lesions develop the polycythaemia does not recur.

Yoffey: Was it from the cerebellar tumour itself that the erythropoietin was extracted?

Stohlman: It was obtained from the fluid in a cystic tumour; the cerebrospinal fluid, blood and urine were negative. I think the characteristics of the material were similar to that of erythropoietin, which we gave to the patient from a child with aplastic anaemia

Van Dyke: The tumour was not removed—just the cystic fluid.

Stohlman: But the fluid was active.

Yoffey: I really wanted to clarify that because via the tentorial

Dr. Van Dyke, one thing that concerns me is that you saw a 55 per cent increase in red cell mass in monkeys but no increase in haemoglobin and haematocrit. Clearly there also must be an enormous increase in the plasma line. Is there an explanation for this?

Van Dyke: We did not do simultaneous plasma volume measurements but one must assume from the labelled red cell dilution measurements either that the plasma volume increased by 50 per cent or that the sample drawn did not represent the average body haematocrit.

Stohlman: Dr. Goldwasser and Dr. Jacobson suggested that cobalt was something which was readily available to all of us and that we might standardize our assays in this fashion. It is not quite as desirable as having a standard product, particularly because cobalt is a toxic compound and if you give too much of it you kill the animal. Moreover I am not certain that we are necessarily measuring the same effect with cobalt as with other materials.

I don't know how many people are familiar with the fasted assay. Normally you starve the animal on day 1 and 24 hours after that, on day 2, you inject erythropoietin; you repeat the injection on day 3, then on day 4 you inject iron, and on the fifth day or 18 hours later you determine the iron uptake. We varied this regimen—this is only a single experiment. We started starving at the usual time and injected on the morning of the third day; six hours later we gave iron. Eighteen hours thereafter we sampled and the values were: normal, 10 per cent incorporation; recipients of extracts of urine from a patient with aplastic anaemia, 18 per cent; altitude plasma 17 per cent. In the routine five-day assay the values are 7, 15 and 27 per cent respectively. I don't see how these results could be accounted for by increased stem cell differentiation alone. It seems that in addition there must be an accelerating effect. We did not test the cobalt with these groups but it is possible that all material may not give the same response as cobalt. In fact the response is quantitatively if not qualitatively different in this experiment. Consequently I think a standard preparation of erythropoietin might be better.

Jacobson: I would be very happy to have direct proof on whether or not the kidney is involved in erythropoietin production. Our evidence that it is involved in production is indirect.

Gordon: T. A. Waldmann and E. H. Levin at the Cancer Institute

appear to be elaborated by the marrow, increased plasma activity would then be expected in anaemic states due to blood loss whether from haemolysis or bleeding and in patients with impaired erythrocytogenesis secondary to well-established marrow deficiences or abnormalities. The latter would include such disorders as leukaemia, iron deficiency, pernicious anaemia, the haemoglobinopathies, certain types of marrow hypoplasia, etc. Although the number of patients studied to date has been small and different parameters have been used to demonstrate erythropoietic stimulation, we have detected increased plasma activity in all of these anaemic states. Patients with anaemias of unknown aetiology, e.g secondary to uraemia, may, however, present a different problem since alterations in humoral factors may be of pathogenic significance Your frequent finding of no increase in humoral erythropoietic activity may reflect the difficulties inherent in the methods available to demonstrate changes in erythropoiesis, or it may be that these clinical states are not associated with increased plasma erythropoietic activity. If these patients do not have augmented plasma factor activity, I, at least, will have to revise drastically my current concept as to the rôle of the humoral mechanism in red cell production

Lajtha: Dr. Gordon introduced the concept of normal or abnormal tissues developing a metabolic disorder, and causing erythropoietin production. If that is so we probably could exclude anoxia as the particular metabolic disorder in tumour tissue, because that is very common in most human and animal tumours. They have a necrotic centre when they outgrow their capillary supply, and there is good evidence from radiation studies that parts of such tumours are quite severely anoxic. So if there is a metabolic disorder it is not primarily connected with local tissue anoxia—unless it is in a particular target organ.

Stohlman: With regard to the urinary assays, our experience has been somewhat similar to that of Van Dyke, with perhaps the possible exception of a higher percentage of positive urines in hypoplastic anaemias. We have run into one disconcerting thing In one patient with Blackfan-Diamond's syndrome we have performed perhaps 40 urine assays, and there is a rough correlation between the peripheral haemoglobin and the amount of erythropoietin, but it is a very rough

notch you are not so terribly far away from the hypothalamic region I did not know whether it was actually in the tumour or had been found in the blood stream.

Gordon: I am under the impression that Waldmann measured only iron incorporation and reticulocyte numbers as his criteria for erythropoietic stimulating activity of the cerebellar tumour fluid.

Stohlman: He measured the iron incorporation in the fasted rat. He did not have very much of the fluid.

Gordon: We have made organ extracts and found them occasionally to be haemolytic.

Stohlman: I am reasonably certain that Waldmann and Levin measured the haematocrits in the animals and the haematocrits did not go down.

Gordon: It is important to measure other parameters besides iron incorporation and reticulocyte numbers in order to preclude haemolytic effects.

Stohlman: I talked to Waldmann before he did it and described in detail the assay method we use. We normally do haematocrits and I suspect he did this.

Gordon: Dr. Van Dyke, would you clarify your discussion on the parabiosis experiments? Was iron incorporation measured in both the hypoxic nephrectomized partner and the normal one not exposed to hypoxia?

Van Dyke: Because of the mixing of the blood, the ^{59}Fe red cell incorporation has to be done for the pair as a unit rather than in the individual animal. A footnote in the table indicates to which member of the pair the iron was given. Under these conditions it makes no difference whether the iron was given to the normal or nephrectomized, hypoxic or normoxic, and it makes no difference from which member the sample is taken for counting.

Linman: I am somewhat disturbed by your Table I because of its clinical implications. The donors, except for those [illegible] tumours and polycythaemia [illegible] knowledge [illegible] the [illegible] logical to surmise that hun[illegible] most types of anaemia would have enhanced plasma erythropoietic activity. Since the humoral factors do not

appear to be elaborated by the marrow, increased plasma activity would then be expected in anaemic states due to blood loss whether from haemolysis or bleeding and in patients with impaired erythrocytogenesis secondary to well-established marrow deficiences or abnormalities. The latter would include such disorders as leukaemia, iron deficiency, pernicious anaemia, the haemoglobinopathies, certain types of marrow hypoplasia, etc. Although the number of patients studied to date has been small and different parameters have been used to demonstrate erythropoietic stimulation, we have detected increased plasma activity in all of these anaemic states. Patients with anaemias of unknown aetiology, e g. secondary to uraemia, may, however, present a different problem since alterations in humoral factors may be of pathogenic significance. Your frequent finding of no increase in humoral erythropoietic activity may reflect the difficulties inherent in the methods available to demonstrate changes in erythropoiesis, or it may be that these clinical states are not associated with increased plasma erythropoietic activity. If these patients do not have augmented plasma factor activity, I, at least, will have to revise drastically my current concept as to the rôle of the humoral mechanism in red cell production.

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Stohlman. With regard to the urinary assays, our experience has been somewhat similar to that of Van Dyke, with perhaps the possible exception of a higher percentage of positive urines in hypoplastic anaemias. We have run into one disconcerting thing. In one patient with Blackfan-Diamond's syndrome we have performed perhaps 40 urine assays, and there is a rough correlation between the peripheral haemoglobin and the amount of erythropoietin, but it is a very rough

correlation. On one or two occasions we found no activity when the haemoglobin was as low as 2·5 g. Whether this means that at this particular time something interfered with the production mechanism or not, I don't know. Certainly before saying that a urine is positive or negative I think one should try it on two or three occasions. In the haemolytic disorders we have not found erythropoietin at all, unless the haemoglobin gets quite low, as with thalassaemia major. Using this and some of the notions we developed from studies of turnover of erythropoietin in animals we suggested that there was utilization by the bone marrow (Stohlman, F., Jr., and Brecher, G. [1959]. *Proc. Soc. exp. Biol. (N.Y.)*, **100**, 40), in which case the greatest activity would be seen in those instances where there was little utilization, e.g. aplastic anaemia, but it would be hard to find in the presence of an active marrow such as in a haemolytic disorder.

TRANSFUSION-INDUCED POLYCYTHAEMIA AS A MODEL FOR STUDYING FACTORS INFLUENCING ERYTHROPOIESIS

L. O. JACOBSON, E. GOLDWASSER and C. W. GURNEY

[illegible]*

Our entry into the study of the control of erythropoiesis was originally related to our continuing interest in the problem of recovery from radiation injury, and we naturally investigated any possible means of stimulating the haematopoietic system. Our first problem was to design an assay system for the plasma erythropoietic factor(s) (hereafter referred to as erythropoietin) that required neither a vast amount of time or effort, nor a large volume of plasma or plasma extracts for a single assay.

Until 1955 assay for erythropoietin was confined largely to the study of the erythropoietic response of normal rodents and rabbits. A positive erythropoietic response to repeated plasma injections was considered to be demonstrated by an increase in reticulocyte, haemoglobin, and erythrocyte values of the peripheral blood, an increase of the red cell mass, or histological evidence of bone marrow hyperplasia. Simultaneous use of all of these parameters was usually not employed by all investigators.

This general approach to assay was originally introduced by Carnot and Deflandre (1906), and with certain modifications continues to be useful. If these parameters are employed the erythropoietin titre of the sample being tested must be high, in

* Operated by the University of Chicago for the United States Atomic Energy Commission.

order to obtain unequivocal differences from control preparations. In 1955 we demonstrated that injection of active plasma into normal rats would elevate the rate of iron-59 incorporation into the circulating red cells. As a regular assay procedure we administered three 2 ml. plasma injections on three successive days; on the third day, a few hours after the last plasma injection, 1 μc of ^{59}Fe citrate was injected intravenously, and 18 to 20 hours later a 1 ml. sample of blood was withdrawn and counted in a well-type crystal scintillation counter. Results were expressed as a percentage of injected counts per minute incorporated into the total red cell mass (Plzak *et al.*, 1955). By this method, control rats given normal plasma or saline had a ^{59}Fe red cell incorporation of about 35 per cent, whereas the rats injected with active plasma had values of 45 to 50 per cent. Although this assay method was much less time-consuming, required relatively little plasma for test purposes, and was reasonably reproducible, it had one of the serious disadvantages that was true of the other assay procedures in use at that time; namely, *relative insensitivity*.

The influence of hypophysectomy on erythropoiesis

[illegible]

undertook the study of the hypophysectomized rat in order to determine the mechanism of this reduction in erythropoiesis We found that within two weeks after hypophysectomy of the adult rat the reticulocyte value and the ^{59}Fe red cell incorporation of the peripheral blood was reduced by a factor of nearly ten, and remained thus depressed for several weeks. These hypophysectomized rats with a markedly reduced rate of erythropoiesis showed increased responsiveness to the injection of exogenous erythropoietin. The reticulocyte response and the ^{59}Fe red cell incorporation of these animals injected with anaemic plasma was several-

fold greater than that observed after normal plasma or saline injection (Fig. 1).

This exciting observation led us to examine the question of whether the hypophysis produced a substance that acted directly on erythropoiesis. We were able to demonstrate that the plasma of hypophysectomized rats made anaemic by bleeding after hypophysectomy contained an increased titre of erythropoietic activity,

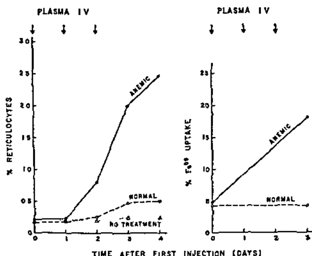


FIG. 1. Assay of anaemic plasma in hypophysectomized rats.

comparable to that observed in normal rats subjected to phlebotomy (Fried *et al.*, 1956). Gordon had previously reported that reticulocytosis would appear in hypophysectomized rats in response to haemorrhage (Gordon *et al.*, 1955). These results in our own laboratory and those of Dr. Gordon largely eliminated the hypophysis as a source of an erythropoietic substance with a direct action on erythropoiesis—a theory which had previously been advanced and subsequently withdrawn by others (Van Dyke *et al.*, 1954).

In an attempt to explain the rapid reduction in erythropoiesis and the marked responsiveness of the hypophysectomized rat to exogenous erythropoietin, we postulated that *upon loss of the pituitary hormones after hypophysectomy, the metabolism of the animal fell rather abruptly to a lower level and thus the overall metabolic oxygen requirement was reduced.* The red cell mass, on the other hand, fell slowly after hypophysectomy; and thus before the red cell mass reached its new, and lower, equilibrium, the oxygen-carrying capacity or supply was greater than required. Consequently, production of erythropoietin fell to a much lower level than normal and the rate of erythropoiesis was reduced accordingly. This postulate was strengthened by the observed responsiveness of the hypophysectomized animal to exogenous erythropoietin and suggested that erythropoietin production was controlled by the relationship between the supply of oxygen available to the tissues of the body and the tissue demand for oxygen. We were thus led to consider other experimental conditions in animals in which either the oxygen demand was increased without a change in the oxygen supply, or in which the oxygen demand was unchanged but the oxygen supply was increased. Accordingly, we studied hyperoxia (Goldwasser, Fried and Jacobson, 1958), acute starvation (Fried *et al.*, 1957) and hypertransfusion (Jacobson *et al.*, 1957) and found that in each of these conditions erythropoiesis was rapidly and drastically reduced, while responsiveness to exogenous erythropoietin was markedly increased. The increased erythropoietic response to erythropoietin administration in each of these conditions was measured by the reticulocyte increase in the peripheral blood or by an increase in ^{59}Fe red cell incorporation. These observations provided us with illuminating information on the mechanism of the overall control of erythropoiesis, as well as with several relatively simple assay methods for erythropoietin. As our experience with these methods has increased, we have found that the most reliable of these assay methods used the acutely-starved rat and the transfusion-induced polycythaemic

mouse. Attention should be directed to the work of Crafts and Meineke (1957), who showed that the oxygen requirement of the hypophysectomized rat is actually reduced. This work lends support to our original hypothesis on the mechanism of suppression of erythropoiesis in this condition (Fried *et al.*, 1956).

Preparation and methods of use of the polycythaemic mouse for erythropoietin assay

As stated above, the most sensitive test for erythropoietic activity of which we are aware is the transfusion-induced polycythaemic mouse. The strain of mice used and the weight or age are not important factors, but we regularly use the female CF No. 1 mouse, age about ten weeks, weighing 20 to 25 g. Our routine preparation of the mouse for assay involves the intraperitoneal injection of 0 5 ml. of washed, packed, homologous red cells on three consecutive days, and repeated on day 5. At the end of this time, the haematocrits of the mice are in the neighbourhood of 75 per cent. By five to six days after beginning the red cell injections, the reticulocytes are at 0·00 per cent, and all evidence of active erythropoiesis in the bone marrow and spleen has ceased. This state can be maintained indefinitely by giving additional red cell transfusions to maintain the haematocrit in the 70 per cent range. Beginning on the sixth day, the plasma or other preparation for erythropoietin assay is given in 0·5 ml. amounts subcutaneously or intravenously for four consecutive days. The reticulocytes of the peripheral blood are sampled daily. Fig. 2 shows the response to an assay of a purified preparation of anaemic sheep plasma. By two days after the initial injection of exogenous erythropoietin erythropoiesis is already active in the marrow and spleen, even though the reticulocytes of the peripheral blood have only reached about 0·2 per cent; the daily injection of the same dose of erythropoietin maintains a relatively constant reticulocyte level in the peripheral blood.

Upon cessation of erythropoietin injections the reticulocyte value returns to zero in three to four days.

Normal plasma and saline are regularly used as controls. Occasionally after four 0·5 ml. injections of normal plasma the reticulocytes reach 0·1 per cent. Saline produces no response.

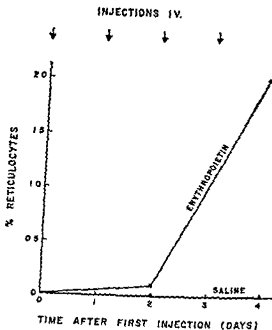


FIG. 2. Reticulocyte response of polycythaemic mice to purified sheep erythropoietin

If, however, normal plasma is continued daily for six to seven or more days, a slight increase in reticulocyte response of 0·1 to 0·3 per cent is usually observed. This strongly suggests that sufficient erythropoietin is present in normal plasma to produce a reticulocyte response in this sensitive assay system.

Another method of assay using the transfusion-induced polycythaemic mouse is to begin red cell transfusion and the injection of the

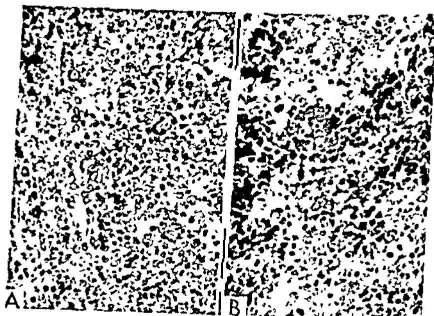


FIG 4. Histological effect of four 0 5 ml injections of rat plasma on splenic erythropoiesis in mice with a transfusion-induced polycythaemia Tissues taken 8 days after first injection of plasma (× 95)

A Spleen of normal plasma-injected mouse Red pulp is devoid of erythropoietic foci

B Spleen of mouse injected with plasma from anaemic rat Marked erythropoietic activity in the red pulp

material for erythropoietin assay on the same day (Jacobson *et al.*, 1957). This is illustrated in Fig. 3. It is of interest to note again that the response to plasma with an active erythropoietin titre levels off at about 2·5 per cent by six to eight days, whereas normal plasma is at about the 0·2 per cent level and the saline controls have fallen to zero. Fig. 4 shows the erythropoietic response of the spleen at eight days.

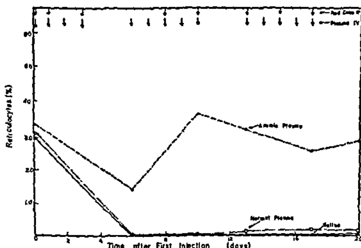


Fig 3 Effect of repeated injections of anaemic and normal rabbit plasma on the reticulocyte values of mice that received intravenous injections of plasma beginning on the same day that transfusions for polycythaemia were initiated

Using either of the techniques described above, one can substitute ^{59}Fe red cell incorporation as the indicator of increased erythropoietic activity. If this is done, then the technique is essentially the same as that used in the starved rat assay, i.e. on the day of the second or third injection of exogenous erythropoietin, 1 μc of ^{59}Fe citrate is given intravenously, and 18 to 20 hours later a 0·1 ml. sample of blood is withdrawn. This sample is

counted in a well-type scintillation counter and the percentage of ^{59}Fe red cell incorporation is calculated as previously described (Plzak *et al.*, 1955).

It is of interest to note that Dahl, Blaisdell and Beutler (1959), working in our laboratories, found that hypertransfusion of the rat almost invariably produced gastric ulceration and haemorrhage. In our original studies on hypertransfusion as a means of suppressing erythropoiesis we observed that it was difficult to maintain the haematocrit at about 70 to 75 per cent in the rat, whereas the mouse presented no special problem, and in no mice have we observed gastric or other gastrointestinal ulceration or haemorrhage. Dahl's observations thus eliminate the hypertransfused rat for erythropoietin assay. We have no explanation of this interesting species difference in response to hypertransfusion.

Some studies using the transfusion-induced polycythaemic mouse

A. The effect of continued polycythaemia on the capacity to re-initiate erythropoiesis

One can easily prolong polycythaemia in the mouse by giving repeated red cell transfusions, as needed, to maintain the haematocrit between 70 and 75 per cent. In this way it is possible to suppress erythropoiesis completely for an indefinite time. We had assumed that if erythropoiesis were thus sustained at zero it would normally appear again when the haematocrit was allowed to fall to or below the normal range. To test this we studied a group of ten mice maintained with induced polycythaemia for three months, and the reticulocyte values of each mouse were found to remain at zero throughout the period. Finally, the haematocrit was allowed to fall and erythropoiesis quickly reappeared when the haematocrit reached the normal range.

B. Transplantation of polycythaemic marrow

We also studied transplantation of marrow and spleen cells from polycythaemic mouse donors to lethally-irradiated recipients of the same strain and found that erythropoiesis began in the recipient at the same time after transplantation as in irradiated recipients which had received normal bone marrow or spleen. In point of fact, there was no evidence to suggest that such marrow transplants had any appreciable, differentially beneficial effect, or the reverse, on growth of any of the cell types of the transplanted tissue (Table I). Although these findings on the capacity to re-initiate erythropoiesis after a prolonged period of polycythaemia and upon transplantation are not unexpected, they serve to illustrate that the red cell precursor can apparently remain dormant for long periods of time, or that erythropoiesis can originate at any time from the mother reticular tissue or multipotential stem cells in response to a suitable stimulus.

C. The effect of an atmosphere containing 10 per cent oxygen on the erythropoietic response of polycythaemic mice

The exposure of normal mice to an atmosphere having a lower than normal concentration of O_2 produces an increase in erythropoietin titre and an erythropoietic response comparable to that observed in rats similarly exposed. Stohlman and Brecher (1957) have shown that exposure to a simulated high altitude (hypoxic anoxia) for 8 to 24 hours increases the plasma erythropoietin titre of rats.

Because of our interest in the mechanism of erythropoietic suppression in the polycythaemic mouse we studied the effect of an atmosphere with a 10 per cent oxygen content on the erythropoietic response of transfusion-induced polycythaemic mice. In Table II the effects of exposures of groups of mice to low oxygen tension for 0, 8, and 12 hours are compared. It is evident that no

Table I

EFFECT OF BONE MARROW FROM NORMAL AND TRANSFUSION-INDUCED POLYCYTHAEMIC MICE ON THE LEUCOCYTE, HAEMATOCRIT AND RETICULOCYTE VALUES OF CF No. 1 FEMALE MICE EXPOSED TO 750 R. TOTAL-BODY X-RADIATION

Time in days	0	3	7	10	14	18	21	24	29
Leucocytes (cu. mm.)									
Controls (untreated)	11,600	10,200	12,100	10,800	8,750	12,000	18,450	9,750	12,800
Normal bone marrow	15,500	480	1,650	3,450	6,750	8,150	7,900	8,100	8,200
Polycythaemic bone marrow	15,500	435	2,310	3,200	3,350	3,900	7,400	6,800	6,150
X-ray only	15,300	375	430	650	dead	—	—	—	—
Haematocrit (%)									
Controls (untreated)	52	50	52	52	50	53	44	50	52
Normal bone marrow	51	51	45	38	39	41	37	34	36
Polycythaemic bone marrow	51	51	44	35	36	40	42	39	38
X-ray only	51	50	41	21	dead	—	—	—	—
Reticulocytes (%)									
Controls (untreated)	3·0	3·9	5·1	5·9	4·9	4·2	6·7	5·8	4·0
Normal bone marrow	3·1	0 0	3·8	9·8	13·7	10·5	10·1	6·3	8·7
Polycythaemic bone marrow	3·7	0·0	2·3	9 0	16·3	12·6	8·3	7·0	6·0
X-ray only	3·8	0 0	0·01	0·05	dead	—	—	—	—

Table II

EFFECT OF EXPOSURE TO 10 PER CENT O_2 FOR 8 AND 12 HOURS ON THE RETICULOCYTE VALUES OF MICE WITH TRANSFUSION-INDUCED POLYCYTHAEMIA

No exposure to 10 per cent O_2						8-hr exposure to 10 per cent O_2						12-hr. exposure to 10 per cent O_2					
Mouse No	0 hr	2 d	3 d	4 d	5 d.	Mouse No.	0 hr.	2 d.	3 d.	4 d.	5 d.	Mouse No.	0 hr.	2 d.	3 d.	4 d.	5 d.
									Reticulocyte values								
1	0	0	0	0	0·01	11	0	0	0	0	0·01	21	0	0·01	0·02	0·1	0·01
2	0	0	0	0	0	12	0	0	0	0·01	0	22	0	0	0	0	0
3	0	0	0	0	0·01	13	0	0	0·01	0	0	23	0	0	0	0	0
4	0·01	0	0	0	0	14	0	0	0	0	0	24	0	0·01	0·01	0	0
5	0	0	0	0	0	15	0	0	0	0·15	0·01	25	0·01	0	0	0	0
6	0	0	0	0	0	16	0	0	0·03	0	0	26	0	0	0·01	0	0
7	0	0	0	0	0	17	0	0	0	0	0	27	0·01	0	0	0	0
8	0·01	0	0	0	0	18	0	0	0	0	0	28	0	0	0	0	0
9	0	0	0	0	0	19	0·01	0	0	0	0	29	0	0	0·01	0	0
10	0	0	0	0	0	20	0·01	0	0·02	0·1	0·01	30	0	0	0	0	0
									Haematocrit values								
1	78	70	69	63	64	11	77	69	65	64	62	21	73	69	62	57	57
2	77	72	70	59	61	12	75	63	63	66	62	22	74	66	63	55	50
3	79	70	68	61	63	13	72	68	61	61	46	23	70	68	64	64	61
4	72	68	65	57	61	14	76	73	72	70	66	24	74	70	65	65	64
5	74	72	65	63	64	15	73	63	60	55	55	25	74	58	64	65	61
6	76	72	67	64	55	16	74	68	70	54	59	26	68	71	68	55	61
7	75	76	71	65	61	17	77	73	69	69	68	27	75	68	70	61	62
8	78	72	63	62	62	18	78	72	67	65	71	28	78	73	70	62	66
9	76	74	72	66	68	19	81	75	75	71	70	29	80	72	67	67	65
10	75	73	69	69	64	20	80	63	58	61	55	30	78	71	72	67	72

significant reticulocyte response was observed. The most obvious explanation is that the increased red cell mass effectively compensates for this degree of anoxia and therefore no stimulus for erythropoietin production occurs. Another possibility is that the polycythaemic state renders the site of formation insensitive to the anoxia. If the polycythaemic state alters the sensitivity of the site of erythropoietin formation then it must be reversible, since upon rapid reduction of the haematocrit of polycythaemic mice below the normal value erythropoiesis invariably recurs in the expected time of three to four days.

D. The effect of cobaltous chloride on erythropoiesis in the polycythaemic mouse

Goldwasser and co-workers (1958) established the fact that the injection of cobaltous chloride increased the erythropoietin titre in the plasma of rats. It was found that a dose of 5·0 micromoles would produce a minimal erythropoietic response in the starved rat. If the starved rat and the polycythaemic mouse were equally responsive to cobalt ion, then one would expect the polycythaemic mouse to have an erythropoietic response to a daily injection of 0·5 micromoles or less. We felt that a determination of the erythropoietic response of polycythaemic mice to this injection of cobaltous chloride might reveal a minimal reproducible value that would serve as a basis in establishing a unit of erythropoietic activity.

As shown in Table III, polycythaemic mice were divided into groups and given daily intraperitoneal injections of cobaltous chloride ranging from 0·2 to 4·5 micromoles. Reticulocyte counts were made daily but no response was observed for these quantities. In another experiment reported by Krantz, Goldwasser and Jacobson (1959) a dose of 5·3 micromoles for three days increased the reticulocyte count from 0 0 to 0·1 to 0·4 per cent. Administration of this dose for longer periods resulted in death of all the animals. In yet another experiment (Jacobson *et*

Table III

EFFECT OF INTRAPERITONEAL INJECTIONS OF COBALTOUS CHLORIDE ON THE RETICULOCYTE RESPONSE OF CF No 1 FEMALE MICE WITH A TRANSFUSION-INDUCED POLYCYTHAEMIA

Number of mice used	*Amount of* $CoCl_2$ (μmole)	*Days of administration*	*Reticulocyte response*
8	0·2	39	0
10	1·0	24	0
10	1·5	21	0
10	4·5	8	0

al., 1959) we administered a dose of 5 micromoles of cobaltous chloride subcutaneously to a group of normal mice eight or nine hours before we collected the blood. The plasma from these animals, which was essentially devoid of free Co^{++} ion, produced in polycythaemic mice an erythropoietic response (reticulocytes) comparable to that observed from anaemic plasma of known potency (Table IV). Furthermore, it was demonstrated that

Table IV

EFFECT OF FOUR SUBCUTANEOUS INJECTIONS OF PLASMA FROM COBALT-INJECTED MICE, AS COMPARED WITH THAT OF NORMAL MOUSE PLASMA WITH COBALT ADDED, ON THE RETICULOCYTES OF MICE WITH A TRANSFUSION-INDUCED POLYCYTHAEMIA

	Haematocrit (%)		*Reticulocytes* (%)	
Time in days after first plasma injection	*0*	*4*	*0*	*4*
Normal plasma with cobalt added	76	68	0	0
Plasma from cobalt-injected mice	76	64	0	2·5

plasma harvested from rats injected with 250 micromoles of cobaltous chloride produces an erythropoietic response in polycythaemic mice. This fivefold difference in the erythropoietic response of starved rats and polycythaemic mice to cobalt ion is striking and deserves further investigation. It is possible that the polycythaemic state renders the site of erythropoietin production

significant reticulocyte response was observed. The most obvious explanation is that the increased red cell mass effectively compensates for this degree of anoxia and therefore no stimulus for erythropoietin production occurs. Another possibility is that the polycythaemic state renders the site of formation insensitive to the anoxia. If the polycythaemic state alters the sensitivity of the site of erythropoietin formation then it must be reversible, since upon rapid reduction of the haematocrit of polycythaemic mice below the normal value erythropoiesis invariably recurs in the expected time of three to four days.

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Third, that both rat and mouse erythrocytes are produced; and

Fourth, that only mouse erythrocytes are produced.

The responses observed are briefly as follows:

Fig. 5 represents a polycythaemic mouse that received rat bone marrow after exposure to 800 r. total-body X-radiation. The

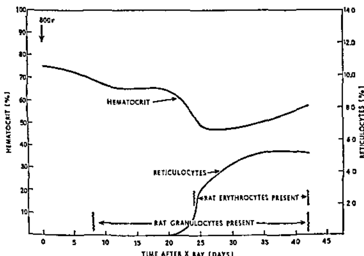


FIG. 5. Response of polycythaemic X-irradiated mouse to rat bone marrow (living 50 days). This mouse produced both rat granulocytes and rat erythrocytes.

haematocrit was maintained above 65 per cent for 18 to 20 days. With the fall in haematocrit, as illustrated, the number of reticulocytes rose. As far as could be determined by alkaline phosphatase staining, granulopoiesis of rat origin had already begun by eight days after the transplantation. This condition still obtained at 42 days It is obvious that suppression of erythropoiesis for 18 days after the rat-cell transplant did not interfere with the formation of rat red cells when the stimulus for erythropoiesis was applied. This suggests that specific rat red cell precursors

relatively insensitive to cobalt ion. On the other hand, we have learned that it is difficult if not impossible to achieve a significant polycythaemia in mice by cobalt ion administration in doses up to 4·5 micromoles for 69 days. This may be due in part to the toxicity of cobalt in mice.

The response of polycythaemic irradiated mice to rat bone marrow

When polycythaemic mice are subjected to a lethal dose of X-radiation some will survive if injected with heterologous bone marrow. This procedure provides a model for the study of the effect of suppression of erythropoiesis on the viability of a heterologous transplant, and of the effect that differentially delaying the growth of a part of a transplant has on the eventual recovery of the haematopoietic system of the irradiated recipient.

Mice (CF No. 1 females) were transfused with washed red blood cells of the same strain. On the seventh day after beginning the transfusions, when the reticulocyte number had fallen to zero, the mice were exposed to 800 r. whole-body X-radiation and 100 million rat bone marrow cells were given intravenously. The red cell transfusions were continued to maintain the haematocrit at about 70 per cent to prevent red cell formation, either by spontaneous regeneration in the tissue of the recipient mouse or from the transplant of rat marrow. The haematocrit could then be reduced at will by bleeding the animal or by allowing natural red cell decay to occur. This allowed us to observe the course of red cell formation as the natural stimulus for erythropoiesis was restored. The results are based on studies in which more than 100 mice were used.

The phenomena relative to erythropoiesis which one might consider operative as the haematocrit falls are:

First, that no erythropoiesis occurs;
Second, that only rat erythrocytes are produced;

cytes began to be produced at the expected time after the transplant. Rat granulocytes apparently disappeared between the 33rd and the 42nd day, and only mouse granulocytes were present. In this instance it is possible that during the period of suppression of erythropoiesis the mouse bone marrow began to resume its

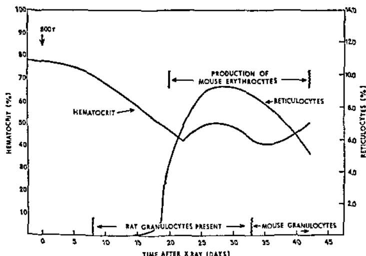


FIG 7 Response of polycythaemic X-irradiated mouse to rat bone marrow (living at 42 days) This mouse produced mouse erythrocytes and rat granulocytes It was sacrificed on day 163 post-irradiation

normal production of mouse red cells It is interesting that mouse granulocytes were not detected until more than ten days after production of the mouse red cells had been restored. One might speculate that in this instance the recipient destroyed the graft This animal was sacrificed 163 days after irradiation.

Fig. 8 illustrates a response that one might expect to encounter occasionally at this irradiation dose level Mouse erythrocytes and granulocytes were produced in the recipient Cells of rat origin were never detected. For some reason, possibly non-specific irritation, regeneration of the mouse haematopoietic tissue

remained dormant for 18 days, or that the production of red cells originated under these circumstances from multipotential rat cells present in the marrow.

Fig. 6 reveals another type of response. In this instance, no additional red cell transfusions were given after irradiation and

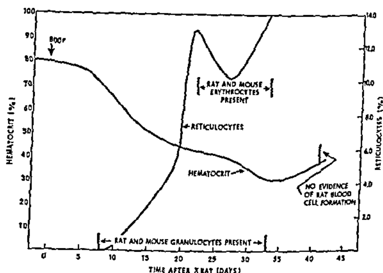


FIG. 6. Response of polycythaemic X-irradiated mouse to rat bone marrow (dead on day 44) In this mouse, both rat and mouse granulocytes and erythrocytes were produced.

rat bone marrow transplantation. The haematocrit began to fall as expected and haematopoiesis was observed by the eighth day post-irradiation. Evidence for production of red cells and white cells of both rat and mouse origin was present. It is of interest but not unexpected that the formation of all rat cells had ceased some time after the 33rd day. Before death this animal presented the usual clinical appearance so characteristic of "late immune" disease, and death may be attributed to the crisis of this syndrome.

Fig. 7 illustrates another variation in response. As the haematocrit fell only mouse red cells were formed, although rat granulo-

Establishment of a unit of erythropoietin

The variability in the erythropoietic potency of purified fractions prepared from the same batch of anaemic plasma but using different methods of extraction and purification is widely recognized. In addition, a marked variability in the loss of erythropoietic activity may and usually does occur even when the same batch of anaemic plasma is processed by the same chemical procedure at different times.

These facts necessitate the arbitrary establishment of a unit of erythropoietin in order that various end-products of plasma processing may be compared. The work of Goldwasser and colleagues (1958) established the fact that cobalt ion produced an erythropoietic response in animals by virtue of the production of erythropoietin. On the basis of this work, Goldwasser and White (1959) studied the effect of single 5 and 10 micromole dosages of cobaltous chloride on the ^{59}Fe red cell incorporation of the starved rat and observed responses substantially greater than in the saline control. They established, in addition, that purified fractions from anaemic sheep plasma gave slopes comparable to that of cobalt in the same response range. Cobalt therefore was accepted as the primary standard for low-level responses, with the 5 micromole response designated as one unit (White *et al*, 1960). At the same time, it is acknowledged that the relationship between the responses to cobalt and erythropoietin may not hold true at higher levels or with different injection schedules.

Site of action of erythropoietin

The difficulty universally encountered in classifying the precursor cells in the blood-forming tissue remains with us. Nevertheless, progress has been made in delineating the site of action of the erythropoietic hormone. Erslev (1959) and Alpen and Cranmore (1959) have concluded, on the basis of distinctly different approaches, that erythropoietin acts on the "stem cells" of

occurred and the mouse survived on this basis. Possibly the effective regeneration of mouse haematopoiesis provided an immunological barrier to the growth of the rat graft.

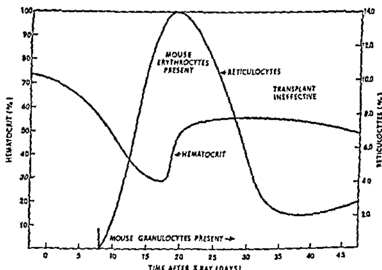


FIG. 8. Response of polycythaemic X-irradiated mouse to rat bone marrow (living at 55 days). No evidence of a transplant was found in this animal.

One may tentatively conclude that (1) "physiological" suppression of erythropoiesis in polycythaemic mice given lethal X-radiation and a rat bone marrow transplant does not interfere with the "take" of the transplant since rat granulocytes generally appear at the expected time, and (2) although erythropoiesis may be suppressed for at least 20 days, upon release of this suppression the production of rat red cells only, of mouse red cells only, or of both may begin. The data suggest that the heterologous transplant contains dormant erythroblasts capable of being activated, or that erythropoiesis may arise under these circumstances from a multipotential cell in the rat marrow transplant.

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the marrow, producing differentiation which culminates in the well-known red cell maturation series. In studying the problem in our laboratory, Filmanowicz and Gurney (1960) have taken advantage of the physiological suppression of erythropoiesis which occurs in the transfusion-induced polycythaemic mouse. In this

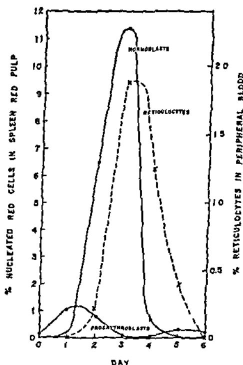


FIG 9 Erythropoietic response in spleen and peripheral blood of polycythaemic mice

preparation, which gives no evidence of active erythropoiesis as judged by the absence of reticulocytes in the peripheral blood and absence of evidence of proliferation in the erythroblast series of the blood-forming tissue, the response to purified erythropoietin previously standardized in cobalt units can be studied after single and repeated injections. The erythroblastic response of the hae-

matopoietic tissue and the reticulocyte response observed in the peripheral blood can be studied simultaneously. As shown in Fig. 9, a single injection of 12 cobalt units of purified erythropoietin produces an orderly differentiation and maturation of erythrocyte precursors with a peak reticulocytosis at three days. The reticulocytes have fallen again to zero by the sixth day after the single injection.

Summary and conclusions

The origin of some current procedures for assay of erythropoietin as they developed in our laboratories is described. Those assay systems that utilize ^{59}Fe red cell incorporation or reticulocytes as a measure of the erythropoietic response include the normal rat, the hypophysectomized rat, the starved rat and the transfusion-induced polycythaemic mouse. The responsiveness of the last three subjects to erythropoietin injection is reflected in the relative or absolute polycythaemia so induced. We have postulated that these artificial states reduce erythropoietin production by virtue of a change in the relationship of tissue oxygen demand to the oxygen supply available. The assay procedure using the starved rat is technically the simplest and least time-consuming; that utilizing the transfusion-induced polycythaemic mouse most nearly represents a physiological suppression of erythropoiesis, and is the most sensitive and reproducible. In addition to its usefulness for purposes of erythropoietin assay, the polycythaemic mouse lends itself admirably to many physiological studies of erythropoiesis.

In the polycythaemic mouse, the entire blood-forming tissue remains apparently devoid of erythropoietic activity, or this function is dormant until exogenous erythropoietin is administered, or endogenous erythropoietin production is restored either by reducing the haematocrit to its normal range, or by the administration of a stimulus such as cobalt ion. The stimulus may act upon any one of a number of cells, including the dormant

the marrow, producing differentiation which culminates in the well-known red cell maturation series. *In studying the problem* in our laboratory, Filmanowicz and Gurney (1960) have taken advantage of the physiological suppression of erythropoiesis which occurs in the transfusion-induced polycythaemic mouse. In this

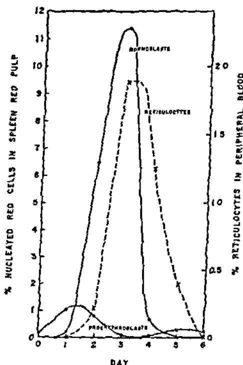


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DISCUSSION

Yoffey: Did you make any differential counts of the marrow you transfused from rats into mice?

Jacobson. Yes, and there are, as one would expect, all the cell types found normally in the bone marrow One can do differential staining to determine whether the growing transplant comes from rat or mouse. On day 3 we can find what we call stem cells in the marrow and spleen. I can also see stem cells during the period when erythropoiesis was suppressed I am not sure I can answer the question you raise, but it seems to me that the stem cells that are in there are really capable of doing almost anything, and that they are now making granulocytes and what have you, but no red cells until the stimulus comes in. The stimulus

primitive erythroblasts, the red cell precursors, and the multipotential cells in the bone marrow.

The bone marrow of these polycythaemic mice, which is apparently dormant or inactive in so far as erythropoiesis is concerned, retains the capacity to initiate erythropoiesis equally as effectively as normal marrow, when transplanted into isologous or homologous supralethally-irradiated mice. Heterologous (rat) marrow may be transplanted to the polycythaemic supralethally-irradiated mouse and suppression of erythropoiesis maintained by post-irradiation transfusion. Such suppression does not interfere with the growth of other transplanted cell types, and the mouse survives. On reduction of the haematocrit or red cell mass to, or below, the normal range, rat red cell precursors multiply and repopulate the mouse haematopoietic tissue. These studies suggest that the isologous, homologous or heterologous transplant contains dormant erythroblasts capable of being activated or that erythropoiesis may arise under these circumstances from multipotential cells in the transplant.

An arbitrary definition of one unit of erythropoietin has been formulated. As a primary standard in the low threshold response range, one unit is defined as the erythropoietic response of the starved rat tissue to an injection of 5 micromoles of cobaltous chloride.

Using the polycythaemic mouse as a subject, the site of action of erythropoietin has been explored and appears to involve principally those undifferentiated precursors which may be proerythroblasts or even still more primitive cells.

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DISCUSSION

Yoffey: Did you make any differential counts of the marrow you transfused from rats into mice?

Jacobson Yes, and there are, as one would expect, all the cell types found normally in the bone marrow. One can do differential staining to determine whether the growing transplant comes from rat or mouse. On day 3 we can find what we call stem cells in the marrow and spleen. I can also see stem cells during the period when erythropoiesis was suppressed. I am not sure I can answer the question you raise, but it seems to me that the stem cells that are in there are really capable of doing almost anything, and that they are now making granulocytes and what have you, but no red cells until the stimulus comes in. The stimulus

could be, of course, bleeding or hypoxia; we have not used erythropoietins in these instances. The differential count of the transplanted material is not really any different from what others have published on the same problem.

Yoffey: In guinea pig marrow we get cells scattered through the parenchyma which we consider to be resting small lymphocytes—whether they are stem cells or not we shall have to argue, but I think Prof. Leblond will agree with me on the cell identification.

Leblond: Yes, they are there.

Yoffey: On the basis of our own studies, the question which at once arises is that in that early regeneration period, if you take sufficiently close intervals, shouldn't you be able to spot these enlarging cells, which we see in our marrow regenerating after irradiation, in the sort of sequence which we have interpreted? If our interpretation is wrong, you might be able to produce a better cell sequence, but at any rate the material seems particularly valuable for picking out the earliest changes in the transformation of the stem cell.

Jacobson: There is no doubt that you can see the beginning of differentiation at 12 hours, and from the histologist's point of view it gives a magnificent opportunity to study the early process of differentiation from stem cells into erythroblasts. We also have smears.

Laitha: If you give tritiated thymidine to the animal, some of these [illegible]

later.

The tritiated thymidine will disappear from the circulation in a few minutes. If you then give erythropoietin, you could observe the appearing pronormoblasts, and note that either (*a*) they are labelled (i.e. come from the large labelled cells), or (*b*) they are unlabelled (i.e. they come from unlabelled small cells). It may be even better to do the experiment with labelled amino acids as these would label 100 per cent of the large cells heavily—you should be able to pick out which is your pronormoblast precursor.

Yoffey: The pachychromatic small lymphocyte, with well marked nucleolus and thick nuclear membrane, we consider to be in the inactive, resting phase. In this phase the cell does not label. If this view is correct, then so long as the polycythaemia is maintained and

stem cells are not needed the small lymphocytes will remain inactive. Should erythropoiesis recommence, however, these cells should enlarge and become leptochromatic, passing through the stage which we have termed the transitional lymphocyte, and then they would label. It would be very interesting to see whether anything of the kind happens in these spleens.

Cronkite: I concur with most of what has been said. Periosteum, for example, which Dr. E. A. Tonna in my laboratory is studying, is an inactive tissue in the adult mouse—mitoses and labelling are rarely seen. However, with the appropriate stimulus, namely a fracture, there is no labelling till about the 10th hour; at 12 hours almost 100 per cent of the cells are labelled. In this two-hour period a mass of cells have gone into DNA synthesis and are commencing the cellular repair. The comment of Dr. Jacobson about observing those cells at 12 hours in the spleen is of considerable interest to us. Perhaps there is enough information on the critical timing to plan the experiment that Dr. Lajtha suggests.

Yoffey: We have had lots of haematological conferences. I would like to have one where we could all get together for a week or two in laboratories with microscopes, and each spend two or three hours looking at one another's preparations. I think it could be very productive, particularly when we have the right type of material.

Gordon: Dr. Jacobson, in your experiments in the polycythaemic mouse, you employed a plasma extract from phenylhydrazinized sheep. You will recall, from our studies, that the activity achievable with higher doses of human urinary erythropoietin is considerably greater than that produced with any level of the animal plasma preparations. I feel, therefore, that your experiments do not necessarily prove that your larger doses of sheep plasma erythropoietin have mobilized all available stem elements for differentiation into the erythroid cell line.

Jacobson: We have not tried anything else; I don't know what would happen beyond this.

Leblond: The excess material, if any, would go to the urine, it would not be stored in the blood.

Lajtha: This is a very important principle. As soon as you differentiate 51 per cent per day of the stem cell population you will run it

down completely (assuming a 24-hour minimum cycle time for the stem cells). So there must be, if one is permitted to think teleologically, a very important safety device for never exceeding 49 per cent.

Yoffey: If one wants to think teleologically in terms of safety devices it is very important to have a considerable excess of stem cells too, so that there will always be a large number to spare. Lymphoid tissue certainly seems to meet a need of that kind, if no other!

Loutit: I think on reflection that the answer you got, Dr. Jacobson, is what one would have predicted. I spoke earlier about the 100 per cent lethal dose and the 60 per cent lethal dose, but that was probably a qualitative type of statement and would have been better phrased in terms of the absolute, the röntgen dose applied, or rads absorbed. Certainly with our own mice there seems to be some sort of critical level at about 800–850 r. We prefer to work well above that, at around 1,000 r., and then we always get consistent results, but if we work at the 800–850 r. level, which in our stock is about the LD_{60}, then we could get the sort of result you got. Would you like to repeat your experiments at 1,000 r.?

Jacobson: It is very difficult to keep these animals alive when you get up to 900—we tried it. If you maintain a polycythaemia such as this, in order to keep erythropoiesis at zero, and then give them 1,000 r. I can tell you that the mortality is going to be so high that you will have attacks of angina pectoris ten times a day, because you feel so badly! The loss even at 800 r. is very great.

Lamerton: Do polycythaemic rats succumb to a lower dose than normal rats?

Jacobson: In the first place 90 per cent of the polycythaemic rats develop gastric ulceration, and therefore you cannot maintain polycythaemia very long without getting into serious difficulty.

Lamerton: What is the mean lethal dose in the polycythaemic mouse as compared with normal?

Jacobson: I have not done it.

Leblond: But you implied that they are more sensitive than the normal?

Jacobson Yes, but I think it has to do with the circulation itself. An expert on sludging might be able to answer this.

Stohlman: It is also a problem to keep them polycythaemic, isn't it?

Jacobson: Yes. You have to retransfuse them.

Yoffey: One other question has arisen recently in connexion with an apparent sudden appearance of these larger cells in a situation where normally only the small lymphocytes are seen. This arises out of G. J. V. Nossal's work (1959. *Brit. J. exp. Path.*, 40, 301) in lymph nodes with the secondary response to antigen. E. H. Leduc, A. H. Coons, and J. M. Connolly (1955. *J. exp. Med.*, 102, 61) have described the explosive appearance of plasma cells, not after the first dose of antigen but after the second dose of antigen about a month later. Nossal looked into that again and found that in about 24 hours you suddenly get large numbers of these primitive reticular cells, which is, I suppose, a name we all understand, but with no mitoses to account for them. He attributed them then to the sudden enlargement of a lot of small cell precursors, in this case small lymphocytes. Dr. Jacobson, have you followed the histology carefully enough in the early stages of this splenic reactivation to say whether you get large numbers of mitoses preceding the appearance of those larger reticular cells?

Jacobson: I don't think there is any doubt that you have an increase, but we have not made counts.

Bangham: During the day I have been collecting "units" of erythropoietin. There is the unit of erythropoietic activity in a preparation of rabbit plasma, made by Borsook, which has been used by many investigators, but which I understand is now nearly finished.

Stohlman: That is not a unit.

Bangham: No, it is a commonly used preparation. People have quoted the activity in terms of this preparation. Cobalt units are apparently widely used, and I think we are in danger of the introduction of batyl alcohol units. Then we have two other kinds which are apparently units of biological effect, one is the haematocrit-cum-reticulocyte unit that Dr. Gordon has been using, and lastly there is a maximal stimulation unit which Dr. Jacobson has just been telling us about. I wonder, in view of this collection of three materials and two activities, whether we could air the question of an accepted standard preparation, it could then be used by all persons assaying erythropoietic activity in order to measure their own preparations. There are certain precepts in biological assay and standardization which I think

down completely (assuming a 24-hour minimum cycle time for the stem cells). So there must be, if one is permitted to think teleologically, a very important safety device for never exceeding 49 per cent.

Yoffey: If one wants to think teleologically in terms of safety devices it is very important to have a considerable excess of stem cells too, so that there will always be a large number to spare. Lymphoid tissue certainly seems to meet a need of that kind, if no other!

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with a potency agreed upon by you, so that all activities can be expressed in terms of the same standard and the same unit.

Yoffey: Thank you very much, Dr. Bangham. This is a very generous offer, and I imagine it is likely that you will be taken up on it.

Cotes: I am concerned about Dr. Jacobson's suggestion that a new unit of erythropoietic activity might be that dosage which would produce the maximum reticulocyte response in polycythaemic mice. Dr. Bangham and I have studied ^{59}Fe incorporation into red cells of mice made polycythaemic by exposure to hypoxia as opposed to transfusion. We find that two strains of mice, Schneider and M.R.C. TO, differ greatly in their responsiveness to erythropoietin derived from plasma of anaemic rabbits or monkeys

Since we all measure erythropoietin by observation of different parameters in various test animals, results from different laboratories can only be compared by reference to the response produced in each test system by a preparation of defined activity. If a standard reference preparation were available for wide distribution it would be extremely useful.

Van Dyke: I think it is absolutely essential to get on with some sort of standard. A few patients who excrete large amounts of erythropoietin could provide enough material to serve as a standard Finding them is going to be a tremendous amount of work. In a city like London you certainly will find a few here and there but it will mean screening hundreds of patients. There are lots of people who have been screened that you don't hear about because of the negative results. There is a great advantage in creating a group whose primary job is to distribute the material they collect Those who are collecting erythropoietin to study its physiology and chemistry are not likely to give it up to be used as a standard.

Stohlman: There is a proposal now under consideration at the National Institutes of Health which might be used for this sort of thing, at least on a small scale

Gordon I would agree heartily with Dr Van Dyke about the advisability of establishing an agency for the collection and distribution of urine containing large amounts of erythropoietin. The availability of active urine depends upon the philosophy of the physician attending the anaemic subjects. In New York City, there appear to be two

it is necessary to follow. It is necessary to assay like with like no matter how crude or apparently non-specific the material is. One can imagine situations where, using a cobalt unit to assay a biological extract, one may be drastically misled. So if a standard could be used I think it should be as similar as possible to the product(s) you are assaying. On the other hand it is equally important, if there is any suspicion, let alone any evidence such as Dr. Gordon and Dr. Linman have produced, of more than one biological activity being present or in need of standardization, that the preparation should contain a reasonable modicum of each activity. At the moment we have the "erythrocyte-stimulating factor" (ESF), (it is not yet clear whether this represents two factors, one which stimulates cell division and one which stimulates synthesis of haemoglobin) and possibly myeloid-stimulating activity. It does not matter, to begin with, if the standard contains both.

Some good evidence has been worked out by Dr. Cotes that even between two species of mouse you can get fairly widely differing responses to the same preparation. With regard to what sort of preparation is desirable, it seems from all that has been discussed that one has to choose between plasma and/or urine Of the preparations from plasma available one can choose one from anaemic rabbits or one which I understand Dr. Jacobson and Armour Laboratories are making from a large number of anaemic sheep. Alternatively, since again it has been stressed that some human urine contains a high concentration of ESF, perhaps one should consider making a collection of a large amount of active urine and processing this to establish material for a standard. These things have been done, for instance with the international reference preparation for human menopausal gonadotrophin 3,500 litres of urine were processed. This was no small task since at the time it was collected there was a petrol shortage and rationing in England. If we could find patients with highly active urine, I am sure we could arrange for its collection and the preparation of an extract which could then be available for any person interested in this field to assay their preparations against. If there is anything which the Department of Biological Standards at Mill Hill can do to help in this way, we would be very pleased to, we have done it for other unofficial standard preparations, in other fields.

I am simply making a plea that someone should prepare a standard

GENERAL DISCUSSION

Reinhardt: Dr. Cronkite's and Prof. Leblond's statements regarding the possible normal persistence of the thymus in man, beyond the usual age of what is considered to be the involutional period, may be compared with work done by me in the monkey in Indonesia. Approximately 35 monkeys of each sex, varying in body weight from that of the newborn to 8 kg., were used. With the increase in age of the individuals, as demonstrated by the body weights (I have no actual data on the age of the animals), there was a continuous fall in the thoracic duct output of lymphocytes which was initiated at a body weight of between 2 to 3 kg, that is, about the period of sexual maturation This is evidence that in this primate form there is a lymphatic tissue involution occurring, as indicated by this type of measurement.

Leblond: Did you obtain the thymus weight?

Reinhardt: Unfortunately no. It is of interest that the thoracic duct lymphocyte output (per kg body weight) of the presumably normal monkeys fits in approximately at the same level as for man. The data for the human are derived from the work of H. R. Bierman and associates, in patients suffering from terminal disease not of haematological origin. Apparently there is a close relationship between thoracic duct lymphocyte output in man and monkey The output in man, monkey, and dog may be contrasted with that for rodents (rat, mouse, hamster). The thoracic duct lymphocyte output in rodents is at a much higher level *in terms of relative body weights* Although there are many parameters to consider, I think the evidence indicates that there is a definite gradient from the rodent through other animal forms, indicating the existence of a significant difference in the number of thoracic duct lymphocytes entering the blood stream in each of these animal forms.

Leblond Were you able to relate this to body surface?

Reinhardt: I did not try.

Yoffey. If you compare the monkey and man which are so closely alike in other ways but very different in body surface, the thoracic duct

points of view. One is that the child with thalassaemia major should not be allowed to fall too low and should be transfused when he or she reaches 5 or 6 g. haemoglobin levels. The second view is that the child should not be transfused too frequently because of the attendant splenomegaly and haemosiderosis. It is from institutions subscribing to this latter opinion that we have been able to collect consistently active urines from children with haemoglobins in the vicinity of 4 g. and below. Calculations indicate that over a period of a year only about six of these highly anaemic patients would be required to make available the same number of units of material as 1,500 phenylhydrazinized sheep.

Stohlman: In fact transfusion to maintain normal haemoglobin or values much above 7 g. may be dangerous since it hastens the day when haemosiderosis will develop, the patients can do quite well at the lower levels of haemoglobin.

out any increased granulopoiesis, presumably. I take it there was no increased neutrophil count in the peripheral blood?

Gordon: Not noticeably in the peripheral blood. In the marrow, as I mentioned, the numbers of myelocytes as well as segmented granulocytes are increased. We intend soon to determine with isotopic methods whether granulopoiesis is actually accelerated in these marrows.

Everett: Some work at present under way in my laboratory is somewhat gratifying for those of us who have been concerned at times about the metabolic stability of DNA labelled with tritiated thymidine. We have obtained large numbers of labelled neutrophils from blood of rats by the sedimentation technique, and placed these in Millipore chambers which were then introduced into the body cavity of the same animal. We are gratified that our grain counts remain the same throughout the period that they survive—24 hours being the longest that I recall.

We had thought that this might contribute to discerning the possible lifespan of the neutrophil, but they seem to live longer here than they do when circulating. Of course they are not subjected to the same conditions and are not battered around perhaps as they are in the blood stream.

A few people have asked about the staining of granulocytes in radioautographs of bone marrow. The Leishman-Giemsa stain is very good to provide for the identification of these cell types.

Jacobson. In the spleen-shielding experiments, when blood formation is, in a sense, rushing into the spleen, it seems to take precedence over everything and the white pulp for all practical purposes will disappear. This may be a completely competitive thing, I have never really explained it. This is also true in a normal animal if you push erythropoiesis as hard as you can with erythropoietins—the splenic lymphatic tissue will be remarkably reduced. In fact you can see erythropoiesis coming up right in the middle of the follicle and the follicle will finally disappear, or just a remnant will be left. I had the evidence that right in the middle of the follicle there was erythropoiesis, but I wasn't smart enough at the time to realize that precursors could have travelled there and set up shop. In the mouse the spleen happens to be a marvellous organ because if you can push erythropoiesis in the

lymphocyte output seems to be rather greater in the monkey, though the difference is not as great as one might have expected. We have thought a great deal about this variation in lymphocyte production. There may be a rough correlation with the life of red cells, but that brings one back to the old problem: if the lymphocyte is a stem cell this makes sense; if it isn't a stem cell why is there a difference?

Cronkite: It is interesting that the two animals at the lower end in their output are the ones that normally have a low steady-state concentration of lymphocytes in the peripheral blood whereas all of the other animals have 60 per cent or more of lymphocytes in the peripheral blood. Perhaps dog and man are similar and the monkey is like all the rest of the smaller animals in respect of the differential count.

Yoffey: Yet the thoracic duct lymphocyte output in the monkey is not so very different from the human.

Trowell: One could say there is a rough correlation with the basal metabolic rate of the whole animal, which is now known to depend on the fundamental built-in rate of oxygen consumption of the tissues. It is in fact correlated with the actual cytochrome oxidase content of the cells, which is inversely related to animal size. I cannot see that it means anything, but the correlation does seem to exist.

Gordon: We have been impressed with the findings reported by Dr. Jacobson and by others regarding the marked depression of erythropoiesis in animals transfused with homologous red cells. Mr. J. LoBue, Mr E S Handler and I have pursued the changes occurring in the leucocyte elements within the marrows of the transfused polycythaemic rat. We have found that the disappearance of the nucleated erythroid cells is accompanied by an absolute increase in the numbers of both young and mature granulocytes. Lymphocyte numbers decrease but not as greatly as the erythroid elements. In other words, the total mass of nucleated erythroid tissue disappearing is replaced by an equivalent mass of granulocytic tissue. This is an interesting phenomenon and I am wondering whether depression of one particular cell line might not set up a signal, possibly humoral in nature, which causes the pluripotential stem cells to proliferate along another cell line.

Trowell: May it not be just an increased storage, a space factor? You could have an increased number of granulocytes in the marrow with-

I have come to think that there is only one sort of reticulum cell, not two; and that it is the ordinary phagocytic reticulum cell which differentiates into the large lymphocyte (lymphoblast). In these cultures one can see all stages in the transition of typical macrophages, containing ingested debris of pyknotic small lymphocytes, through to large lymphocytes, including cells which could be called large lymphocytes but for the fact that they still contain a little debris phagocytized at an earlier stage. This seems to prove that one and the same cell can be both phagocytic and lymphopoietic. Admittedly this is *in vitro*, and I would not claim that this is necessarily the normal mechanism of lymphopoiesis, but the mechanism does exist. If I may contribute to the discussion of these mysterious stem cells in general, I would like to put forward the simple view that they are just ordinary common-or-garden reticulum cells—acting under the influence of certain hormones in certain locations.

Lajtha: Regarding these reticulum cells that Dr. Bessis says transfer iron to pronormoblasts, I don't think there is any evidence that normoblasts get *most of their iron content* from reticulum cells. I want to emphasize that this phenomenon exists, but it is open to question whether the arrow points from the reticulum cells to the normoblasts, *or the other way round In any case the phenomenon is responsible* only for a very minor fraction of the iron administered After a single injection of ^{59}Fe, within minutes, or as soon as you can take the specimen, all the early normoblasts will be labelled and very few of the reticulum cells will be labelled, although if you take such reticulum cells *in vitro* they are quite capable of iron uptake

Braunsteiner That is correct, but normally *in vivo* the organism does not get an injection of ^{59}Fe. It breaks down its own red cells in the reticulum cells You can have the same phenomenon with reticular cells and plasma cells, for instance you get the debris of phagocytized material and very close you get the plasma cell precursors which get grains from the reticular cells and then build up their ergastoplasmic structure. It seems a very common thing.

Lajtha. You can take marrow cells *in vitro* without reticular cells and these cells are quite capable of taking up iron from the surroundings.

Braunsteiner I think they are, but in the normal processes in man they might take it from different sources.

animal beyond the bone marrow capacity erythropoiesis will increase in the spleen.

Yoffey: But what happens to the other lymphoid tissues, the mesenteric lymph node and thymus?

Jacobson: I don't think they are affected.

Yoffey: A few of us, including Prof. Reinhardt, were engaged in some hypoxia experiments on the Jungfrau. We found—again whether there was any correlation or not I don't know—that there was quite definitely an increase in thoracic duct lymphocyte output at that high level (Reinhardt, W. O., and Yoffey, J. M. [1956]. *Amer. J. Physiol.*, 187, 493). You could explain this in several ways: anoxia is known to cause a marked increase in capillary permeability and there might have been an increased flow of lymph, though we did not notice an actual increased lymph volume, washing out free lymphocytes. Unfortunately we did not have time to follow that as far as we would have liked.

Astaldi: Dr. Jacobson, were you able to observe in the centre of your erythroblastic areas the presence of those macrophagic or histoid cells that Bessis called "nutritive cells" and which he showed to be devoted to transferring the ferritin molecules into the erythroblasts?

Jacobson: We are doing some work with the electron microscope at the moment. We think we see essentially the same as Bessis saw.

Astaldi: That is what G. Di Guglielmo showed many years ago in Di Guglielmo's disease. He was able to show these histoid cells in the centre of the erythropoietic areas and interpreted them as histoid pro-erythroblasts. Now Bessis has reconfirmed their existence and he has also shown their function of transferring iron into the erythroblasts. In your central cells, were you able to observe something like that?

Jacobson: I think this gives a splendid opportunity to study the stage at which this process presumably occurs, as Bessis has described it. But we have not gone that far.

Braunsteiner: There is a special form of reticular cell, a cell which distributes the iron particles, but obviously does not become a pro-erythroblast or whatever. That is what I asked Prof. Leblond: are there reticular cells which have the special function of phagocytizing and redistributing material, and which are completely different from the other so-called reticular stem cells?

Trowell: From observations on whole lymph nodes cultured *in vitro*

have mostly been investigated by the tissue culture method in my laboratory, but the problem of haemopoietic differentiation may perhaps be investigated in a new way. Using these humoral factors such as erythropoietin and leucopoietin, and others, which have been discussed here, we may try to obtain haemopoietic differentiation *in vitro*, starting from undifferentiated cells, from small (and why not large?) lymphocytes, and others. A careful and prolonged study by this method may provide us with further experimental data on the problem of the stem cell compartment, in order to show what are stem cells and what are not: histoid cells, lymphocytic cells, and so on.

Yoffey: It has been suggested that I am trying to "sell" the lymphocyte. I assure you I am not! I hold no strong views on the lymphocyte, but all that has happened is that the trend of a number of experiments, whatever we do, seems to implicate the lymphocyte as a stem cell. If someone can produce another stem cell convincingly I should be delighted. I do wish someone would conclusively settle the problem one way or another I have been listening hopefully all the time for stem cell suggestions, and they are still vague. The nearest I have got is a suggestion about percentage Dr Lajtha gave it as 3 to 4 per cent in human marrow. I suggested that in the marrow of smaller animals it should be double or even a little more In that case you have an appreciable number of cells If you sort out myelograms and try to identify a stem cell group constituting 4 to 8 per cent of the total marrow cells you can rule out an awful lot of cells. There are not many cells besides the lymphocyte left to consider If it is true that the stem cell is multipotential and can go on to erythropoiesis or granulopoiesis, then I would like to ask someone like Dr. Gordon to bombard the stem cell from both sides, to give prolonged and intensive dosage with both his neutropoietin and erythropoietin, of which he apparently has a very rich supply, and see what happens It ought to be possible to exhaust the stem cell by very powerful stimuli. I don't know whether the mixed leukaemias, or polycythaemias and leukaemias combined, are not for example due to that sort of combined stem cell stimulation. That is an experiment which on paper at any rate sounds possible. I would like therefore to suggest that intensive stimulation of a possible stem cell from both directions should be tried.

Lajtha: Quite; they get it from the serum—there is a perfectly good serum iron pool.

Cronkite: I think there can be little doubt that what Bessis shows as a phenomenon really exists. The thing that is necessary experimentally, and I believe this is probably the case with ^{59}Fe, is that it should be introduced into the pool in a form and in an amount that does not perturb the normal steady state, or you may be studying not what is going on *in vivo* under ordinary circumstances, but a perturbation of the normal steady state. It is obvious, Dr. Braunsteiner, that between your observations, Prof. Leblond's and our own, there are apparent problems that are difficult to reconcile. Whereas Prof. Leblond primarily looks at the fixed tissue in sections, we primarily observe blood and the lymph in smears, and have not attempted seriously to correlate it with the tissue. For further studies along these lines, trying to build up an entire model, particularly for the lymphocyte, one should look not only at the tissues of apparent origin, but also at all the compartments in which there is an apparent steady state equilibrium. In this respect, all of us have further work that must be done.

Stohlman I spoke with Dr Bessis at some length about this problem. He feels very strongly that the iron is transferred from the reticular cells to the normoblasts. However, he does not think that this is the main source of iron for haemoglobin synthesis, but that most of it comes from transferrin iron. As to the question of which way it goes I am sure there are some mature cells that are phagocytized by reticular cells. There are also some immature cells, perhaps the product of ineffective erythropoiesis such as Alpen has shown with radioautography (Alpen, E. L. and Cranmore D. [1959]. *Ann N Y. Acad. Sci*, 77, 753). The reticular cell may therefore obtain its iron either from the normoblast or from the mature red cell by phagocytosis, and then in turn transfer it to the developing cells. Thus the reaction may go in either direction within the bone marrow. Most of the red cells that are broken down, however, are broken down at sites far removed from the bone marrow digested by reticular cells and the iron is carried back through transferrin.

tions of these tumours? If some lead could be obtained in this area, it would provide important information not only with regard to the production of erythropoietin but other cell proliferation-stimulating agents as well. I am referring to only those cases in which polycythaemia is present.

Rachmilewitz: These are rare!

Stohlman: One interesting area of development might be the direct visualization of marrow after perturbation using a technique such as R Kinosita in the U.S. and Brånemark in Sweden are doing. This would be very helpful. Tissue culture techniques which might support a growth of normal erythroid or myeloid elements over many weeks, months or years would certainly help to supply some answers.

Yoffey: One other problem I wanted to ventilate arises out of the precursor pool in the lymphoid tissue If we take Leblond's suggestion about the pool of eight cells in mitoses, starting from the reticular cell along what we have called the reduction pathway, in the lymphoid tissues there is apparently a one-way change One of the fundamental problems is whether that is an irreversible change or not, or whether the change can ever, at any part of the pathway, start to reverse. The second point, since we know that large numbers of cells leave the reduction pathway at various stages and go out into the lymph, is that if it is an irreversible change then wherever they go each cell represents so many potential small lymphocytes, depending on where it goes, and if it is not an irreversible change there is a possibility of differentiation. One of the questions which further arises is whether the early stages of differentiation can occur in the lymphoid tissue and later changes elsewhere There is suggestive evidence for this. The plasma cell aspect was raised by T Wesslén (1952 *Acta derm -venereol (Stockh)*, 32, 265) when he found his antibody-forming cells in lymph which did not resemble at that stage typical plasma cells. We have heard of Dr. Bessis' finding that some of these cells in lymph may already, with the electron microscope, show the characteristic plasma cell lamellation. One of W. Bloom's experiments (1937. *Anat Rec.*, 69, 99) now falls into line—it was heavily criticised then He gave rabbits repeated injections of *Ascaris* extract and then found the thoracic duct lymphocytes all developing into granulocytes J. Medawar (1940 *Brit. J.*

Everett: It is apparent that one of our biggest problems is to try to get an agreement on what a stem cell is and what its potentialities are along a particular line. It might be feasible, employing the Millipore culture technique, to culture lymphocytes, or whatever cell we are thinking of as a stem cell, within the bone marrow or within what we would think would be the environment that would provide for its expected development.

There also seems to be a need for combining electron microscopy with conventional light microscopy and phase microscopy, and getting down to a basic understanding and appreciation of the finer structure to provide for a basic agreement about these cells. I myself am not happy to accept all electron microscopy without relating it to a conventional standard of what we now call this or that cell.

Yoffey: We all look forward to experiments along those lines. The only thing that worries me about attempts to culture either lymphocytes or any other cells is that you can so readily depart in important particulars from the environment in which the cell normally grows. For example how far might big oscillations in special pressure conditions in the bone marrow, in a rigid cavity, influence cell development? There may be other biochemical factors peculiar to bone marrow, possibly from some of the small amounts of substances in the marrow fat which appear to have special chemical properties. One is

would feel happier about watching the progress of an experiment of that kind

Gordon: Dr. Loutit mentioned that pathology may be a rich source of information about how mechanisms operate under normal conditions and I would like to echo that sentiment

The point was made by me earlier that several tissues may have a latent capacity to produce erythropoietin. Might it not be a profitable venture to ferret out the metabolic lesion which causes tissues like hypernephromas, cerebellar haemangioblastomas and possibly ovarian carcinomas, on occasion, to elaborate erythropoietin? In this connexion, could we induce a biochemical team to investigate possible changes in the enzymes resident in the cytoplasmic microsomal frac-

scopic differentiation of plasma cells and plasma cell-like cells in the lymphatic duct. It is an old custom in clinical haematology to call all kinds of lymphocytes "blood plasma cells". We never found plasma cells in the peripheral blood, or at least not more than one out of I don't know how many. In the thoracic duct, however, there are undoubtedly some real plasma cells which vary in number from zero to two per cent. When we immunize animals, rabbits for instance, after the second immunization we can get a higher number of real plasma cells, not only precursors but fully developed plasma cells These immediately disappear from the blood stream: you can find them in the thoracic duct but you cannot find them afterwards in the blood stream. They might go to the tissue and settle down there.

Yoffey: I agree, but as to the fate of these plasma cells you will find a lengthy paper by A. H. E Marshall and R. G White (1950. *Brit. J. exp. Path.*, 31, 157) in which they show that in hyperimmunized animals you get large numbers of plasma cells and almost the maximum of antibody formation in the lungs. Most of these cells were big cells and the pulmonary capillaries were presumably the first ones in which they were held up

Fichtelius: I have read somewhere that the appearance of plasma cells in the thoracic duct is an artifact. There are plasma cells in the thoracic duct lymph in connexion with bleeding during cannulation. Otherwise you never find plasma cells there. Don't you think it is very peculiar that you cannot find them in the blood, Dr. Braunsteiner, because all of them reach the blood?

Braunsteiner Even if it is irritation they should be found in the peripheral blood during the time of irritation.

Yoffey: I have looked at a fair number of smears of thoracic duct lymph in my time and I think I can state quite definitely that I would be extremely astonished to see the conventional plasma cell as seen with a light microscope. One does on the other hand undoubtedly see the larger basophilic cells which may, under the electron microscope, show the lamellation which you described (Braunsteiner, H., Fellinger, K., and Pakesch, F. [1953] *Blood*, 8, 916) I think when you say plasma cells you mean that?

Braunsteiner: Yes.

Leblond Dr. Sainte-Marie and I feel that the results in the normal rat

exp. Path., 21, 205) repeated it without putting in the *Ascaris* extract and did not get any development of granulocytes. It may well be that already there had been some sort of induction there, not necessarily even from the small lymphocyte stage but somewhere along the earlier part of the reduction pathway. I feel it is time now that thoracic duct lymph began to be analysed by modern methods in terms of different cell groups. By differential sedimentation it ought to be possible to separate two or three different cell groups, with the larger lymphocytes at one end, the intermediate groups in the middle, and even eliminate the small lymphocytes altogether if you felt strongly enough about it. I think most of us are agreed that whatever the small lymphocyte does, the cells on the earlier part of the regeneration pathway are cells capable certainly of DNA synthesis, and possibly of differentiation and development.

Astaldi· For the purpose of separating the different cell groups from the thoracic duct lymph, I should like to suggest experimenting also with Osgood's gradient factor.

Fichtelius. Schooley cultivated thoracic duct lymphocytes with the Millipore filter technique and by adding bone marrow he got plasma cell formation from the medium-sized lymphocytes, but without bone marrow he could not get the plasma cells.

Concerning the separation of different sizes of lymphocytes, I must remind you that we do not know whether the cells which are large in the supravital preparations are the same as those which are large in sections and smears. We have tried to separate these fractions in the two-phase polymer system (Albertsson, P. Å. [1958]. *Nature (Lond.)*, 182, 709) without success, but the experiment with the counter-streaming centrifuge has been more encouraging. We intend to analyse the different fractions with regard to the synthesis of DNA.

Yoffey I only know of one lymphocyte separation experiment, by F. J. Keuning and L. B. van der Slikke (1950. *J. Lab clin. Med.*, 36, 167), with cells from the minced spleen of animals that had been given repeated doses of antigen. They demonstrated that it was the larger cells that had produced the antibody, and that the small lymphocyte group did not produce any

Braunsteiner: We have just published a paper (Braunsteiner, H., and Pakesch, F. [1960]. *Wien Z. inn. Med*, 41, 49) on electron micro-

pounds may be added then to the isolated intact organ systems, which I think could be fruitful if explored.

Yoffey: That would come nearer than any of the tissue culture experiments, I agree.

Trowell: The experience of physiologists has been that such preparations are limited to 12 hours at the very outside—mostly 8 hours. The physiologists have done this for years. Ever since the time of Ludwig it has been one of the basic techniques of experimental physiology, and while there have been improvements in recent years, there are very few organs that can be maintained for as long as 12 hours, so it would be limited to the isotope uptake type of experiment.

Lajtha: Or to the appearance of pronormoblasts such as in Dr. Jacobson's spleens!

Trowell. Yes, I think you could perhaps get through one stage of differentiation and this might be good enough for one or two specific problems. But for anything like normal and sustained haemopoiesis much more stable conditions would be required. Although the perfused organ survives in some shape or form for a few hours, it is in fact going downhill right from the start. In this respect I think the organ culture techniques offer more hope, for although the environmental conditions are still pretty unphysiological, they are at least fairly stable and presumably capable of improvement.

* * *

Yoffey: In closing this symposium I feel, looking round, almost like saying "my dear friends", except that it might make me sound like a clergyman, and I certainly feel no inclination to preach. I want first of all to thank you very much indeed for having come. Many of you have travelled long distances and at great inconvenience, and on behalf of the Foundation, and certainly on my own personal behalf, I am extremely grateful to you. We hope you have found the conference an enjoyable one. Speaking for myself it has been one of the most pleasant conferences I have ever taken part in. We have differed, and differed quite radically, but we have agreed to differ and done it quite pleasantly, and will still be parting the best of friends. I am particularly happy about that because the lymphocyte, of all cells, seems to evoke the most violent emotional reactions. I have recounted how on one

can be explained in a satisfactory manner by assuming that plasmocytopoiesis consists of an irreversible sequence of events; that is, once the evolution of the plasmoblast starts, it keeps on going to the mature plasmocyte stage.

Under certain conditions, the "initial" stem cell might be an immature large lymphocyte, as pointed out above.

Incidentally, Dr. Sainte-Marie believes that plasma cells do not die *in situ* in the medullary cords of thoracic lymph nodes, but migrate outside. This would explain why plasma cells may be seen in so many locations. But how they migrate is not known.

With regard to Dr. Fichtelius's remark about the difficulty of separating the various types of lymphocytes, by centrifugation or otherwise, I discussed with Dr. Astaldi and Dr. Trowell the possibility of cultivating single cells and following their evolution. Technically, this problem seems to be most difficult.

Trowell: I would not say it is not feasible but I think it is certainly going to be very difficult. I have tried on and off for the best part of ten years to achieve some sort of lymphopoiesis *in vitro*. I have tried various systems—isolated lymphocytes as you suggest, whole lymph nodes, different species of animals, thymus and so on, and I have had no success. I have tried all sorts of physical and chemical conditions of the medium, extracts of all different organs, and all the known biochemical stimuli, vitamins and so on. I am sure someone will do it some day, but a lot more fundamental research is needed first. It is easy enough to isolate a single large lymphocyte; the difficulty is to get it to stay alive, let alone to divide or transform.

Lajtha. I would suggest, on the lines you mentioned, Prof. Yoffey, that the milieu of these cells is very important. Perhaps some of our *in vitro* experiments did not succeed because the milieu was not quite right. The milieu of course may not be right even if the tissue is cut up into small bits. There will be a diffusion gradient over the cut surface, there will be the matter of a metal surface touching the cells, and so on. But could we go a little further if we used short-term isolated organ perfusion experiments? I am thinking of keeping intact spleens and such like in organ baths. I realize that in small animals cannulation problems may be difficult, but nowadays recirculation experiments can be done with any medium you like, including the whole blood. Com-

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Plain numbers indicate a contribution by the author, either in the form of a paper or to the discussions

Numbers in italics indicate a reference to the author's work.

occasion—I won't mention names—at a meeting of the American Association of Anatomists I did literally see one anatomist foam at the mouth when it was suggested that the lymphocyte was not a multipotential cell. That was a warning which I have always taken to heart.

I hope that you have enjoyed the proceedings, and that, either from the suggestions for further work which have been thrown out or, what is even more probable, from the off-the-record discussions in the coffee and sherry *intervals*, *ideas for future work have begun to germinate* which will lead to some further solution of this problem that has worried us so very much, the stem cell problem. I hope that if I have an opportunity of meeting some of you again, wherever that may be, we shall be just that little bit nearer to the solution of what is perhaps the most exciting detective story in the whole of modern cytology.

Before I, as Chairman, bid you an official farewell, I would like once again on your behalf to thank Dr. Wolstenholme and his very patient and capable staff for the extremely kind, helpful and efficient way in which they have made the running of this conference possible.

I am now going to declare this sixtieth Ciba Foundation Symposium formally closed.

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